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Downregulation of PARP-1 expression by RNA interference

INAUGURAL-DISSERTATION

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»Avoid any Sympathy for normal People«
(Johan Sjerpstra)

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ADP	Adenosine diphoshate
ATP	Adenosine triphoshate
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
DAPI	4',6-Diamidino-2-phenylindole
DBD	DNA-binding domain
DMEM	Dulbecco`s modified eagle medium
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
ECL	Enhanced chemiluminescence
EDTA	Ethylen diamine- tetraacetic acid
FBS	Foetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H_2O_2	Hydrogenperoxide
HEPES	N-(2-hydroxyl) piperanzine-N`-(2-ethane sulfonic acid)
Kb	Kilo base
kDa	Kilo Dalton
LB Medium	Luria-Bertani Medium
MDa	Mega Dalton
MEF	Mouse embryonic fibroblasts
mRNA	Messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor-kappa B
PAGE	Polyacrylamide gel electrophoresis
PARG	Poly(ADP-ribose) glycohydrolase
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
pSU6	pSilencer^{TM}1.0-U6 siRNA expression vector
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
rpm	revolution per minute
SCE	Sister chromatid exchange
SDS	Sodium dodecyl sulfate
shRNA	short hairpin RNA
siRNA	small interfering RNA
TBE	Tris borate-EDTA buffer
TBS	Tris-buffered saline
TBST	Tris-buffered saline Tween® 20
TBSTM	Tris-buffered saline Tween® 20 plus milk
TRF-1	TTAGGG repeat binding factor 1
v/v	Volume to volume ratio
VPARP	Vault PARP
w/v	Weight to volume ratio

1. INTRODUCTION

1.1 RNA interference (RNAi)

RNA interference is a gene regulatory mechanism that limits transcript levels by activating a sequence-specific RNA degradation process. RNAi and related processes represent evolutionarily conserved mechanisms, which protect organisms from invasion by both exogenous (e.g., viruses) and endogenous (e.g., mobile genetic elements) genetic parasites. In addition, dsRNA-dependent silencing represents a conserved regulatory motif for endogenous programs of gene expression. We are just beginning to understand not only the biological roles of RNAi but also the mechanistic basis of this process.

The first definitive demonstration that RNA could trigger gene silencing came from the discovery of RNAi in *C. elegans*. In an attempt to use antisense RNA to investigate gene function in *C. elegans*, Guo and Kemphues observed that injection of either antisense or sense RNAs into the syncitial germline of worms was equally effective at silencing homologous target genes (Guo and Kemphues 1995). As an extension of these experiments, Mello and Fire tested whether combination of sense and antisense RNAs might enhance the effect (Fire et al. 1998). The result was startling. Combined sense and antisense, in essence double-stranded RNA, was an incredibly potent silencer of gene expression. The dsRNA mixture was at least 10 times more effective than either sense or antisense RNAs alone.

From this discovery emerged the notion that a number of previously characterized, homology-dependent gene-silencing mechanisms might share a common biological root. Several years previously, Richard Jorgensen had been engineering transgenic petunias with the goal of altering pigmentation, but introducing exogenous transgenes did not deepen flower pigmentation: in many of the plants the result was the opposite, white or irregularly coloured petunia petals (Jorgensen 1990). This indicated that not only were the transgenes themselves inactive, but also that the added DNA sequences somehow affected expression of the endogenous loci. This apparent communication between unlinked but homologous loci was termed co-suppression.

Co-suppression phenomena are not restricted to plants: similar outcomes have been noted in unicellular organisms, such as Neurospora, and in metazoans, such as Drosophila, *C. elegans* and mammals (Romano and Macino 1992, Fire et al. 1991, Pal-Bhadra et al. 1997).

Genetic and biochemical studies have now confirmed that RNAi and co-suppression share mechanistic similarities and that the biological pathway underlying dsRNA-induced gene silencing exists, in many, if not most, eukaryotic organisms.

1.1.1 Mechanism of RNAi

The precise mechanisms behind RNAi are not yet fully understood, but in the last few years, important insights have been gained in elucidating key aspects of the RNAi process. RNAi mediated by the introduction of long dsRNA is a two step mechanism (Fig. 1).

The initiation step

Initiation of silencing occurs upon recognition of dsRNA by a machinery that converts the silencing trigger to ~21-25 nucleotide RNAs (small interfering RNAs, siRNA). In Drosophila it was shown that a class of RNase III enzymes produces siRNA from long dsRNAs in an ATP-dependent manner (Bernstein et al. 2001). These enzymes – named DICERS – are evolutionarily conserved in organisms competent for RNAi. Structural information has led to a model in which Dicer functions as an anti-parallel dimer to form ~22mer siRNAs (Hannon 2002). Cleavage into precisely sized fragments is determined by the fact that one of the active sites in each Dicer protein is defective, shifting the periodicity of cleavage from ~9-11 nucleotides for bacterial RNase III to ~22 nucleotides for Dicer.

The effector step

The small interfering RNAs (siRNAs) are signature of RNA interference. They are double-stranded duplexes with two-nucleotide 3`overhangs and 5`-phosphate termini (Zamore et al. 2000; Elbashir et al. 2001b) and serve as a sequence-based template for recognition by RISC (RNA-induced silencing complex), a protein-RNA effector nuclease complex that recognizes and destroys target mRNAs. The siRNAs are incorporated into the multicomponent nuclease RISC. Recent reports suggest that RISC must be activated from a latent form, converting a double-stranded siRNA

to an active form by unwinding of siRNAs (Nykanen et al. 2001). RISC then uses the unwound siRNA through Watson-Crick basepairing as a guide to substrate selection (Hammond et al. 2000) and the specific target mRNAs are degraded.

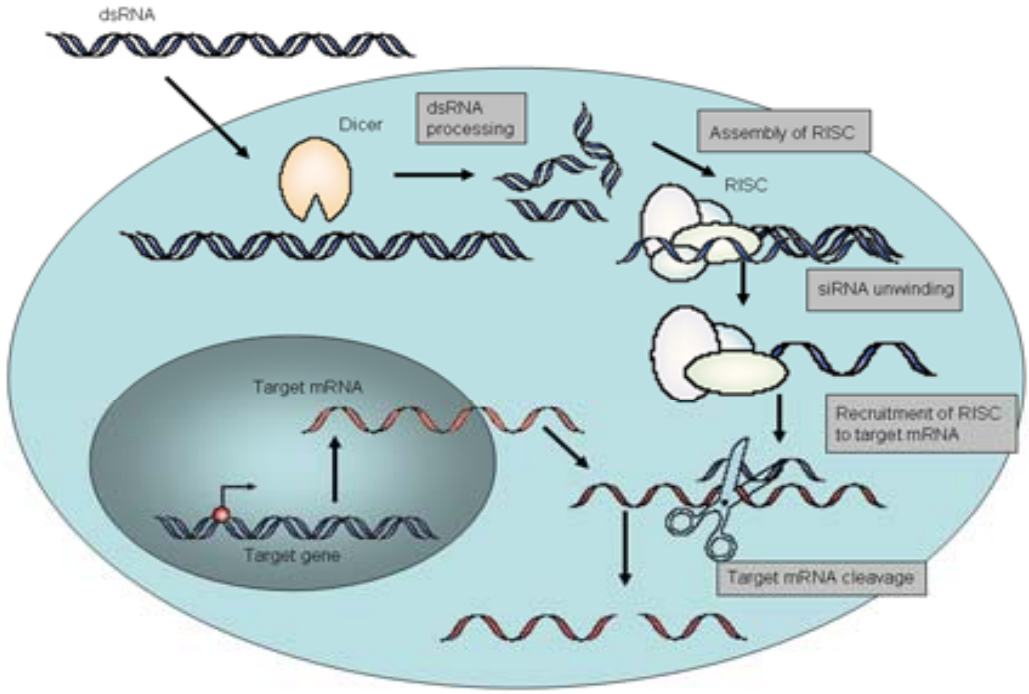


Figure 1: A model for the mechanism of RNA interference.
(Modified from Hannon, 2002)

1.1.2 Systemic silencing and amplification

One of the most provocative and least understood aspects of dsRNA-induced gene silencing is its ability to spread throughout certain organisms. This property is most evident in *C. elegans* and in plants.

In plants the silencing information is transmitted throughout the plant via the plant's macromolecular trafficking system, which is composed of cell-to-cell transport via plasmodesmata and long-distance transport via the phloem translocation system (Palauqui et al. 1997; Voinnet et al. 1998). When the silencing effect is induced by a virus, this process of spreading leads to resistance to the virus in regions of the plant where the virus is not yet active, protecting the plant from further virus infection. But plants are not the only phylum in which systemic silencing effects have been observed.

The effects of dsRNA can also spread to tissues in which it has not been directly introduced in *C. elegans* (Fire et al. 1998). For example, one can feed *E. coli* that produces dsRNA encoded by a plasmid to *C. elegans*. This will lead to a very robust RNAi response not only in the fed animal itself, but also in its progeny (Timmons and Fire 1998).

Obviously, the question regarding systemic RNAi is: How does the dsRNA spread throughout the animal? Are there specialized transport routes for dsRNA, or is it taken up through more general pathways that are also used for the uptake of nutrients? In *C. elegans*, a putative transmembrane protein, SID-1 (systemic RNAi-deficient), was shown to be important for systemic RNAi (Winston et al. 2002). The *sid-1* gene is required to spread gene-silencing information between tissues but not to initiate or maintain an RNAi response. SID-1 homologues are absent from *Drosophila*, consistent with a lack of systemic transmission of silencing in flies, whereas the strong similarity to predicted human and mouse proteins suggest the possibility that RNAi is systemic in mammals (Winston et al. 2002). Further molecular characterization of *sid-1* and other loci will undoubtedly lead to a better understanding of this systemic silencing process.

Screens for genes required for gene silencing in plants, fungi, and worms have identified a family of proteins whose sequences suggest they are RNA-dependent RNA polymerases (RdRPs) (Cogoni and Macino 1999; Dalmay et al. 2000; Mourrain et al. 2000; Sijen et al. 2001). The discovery of RdRPs in RNAi provides a possible explanation for the remarkable efficiency of dsRNA in gene silencing in these organisms. New dsRNA could be synthesized by RdRP and thus amplify the silencing process. In *D. melanocaster* and mammals, RdRP genes have not been identified by database analysis.

1.1.3 RNAi as a tool in mammalian cells

RNAi has evolved into a powerful tool for probing gene function, although it seemed for some time that developing RNAi in mammalian systems would not be feasible because mammalian somatic cells exhibit nonspecific responses to dsRNA which would obscure sequence-specific silencing. One of these is the RNA-dependent protein kinase (PKR) pathway, which phosphorylates and inactivates the translation factor eIF2α, leading to a generalized suppression of protein synthesis and cell death via both non-apoptotic and apoptotic pathways (Clemens and Elia

1997). A second dsRNA-response pathway involving the dsRNA-induced activation of a sequence-nonspecific RNase (RNase L) has also been demonstrated (Player and Torrence 1998).

One way to overcome these nonspecific dsRNA responses is to simply create dsRNA triggers of < 30 bp in length because the activation of PKR by dsRNA has been shown to be length-dependent; dsRNAs of less than 30 nucleotides are unable to aktivate PKR, and full activation requires ~80 nucleotides (Minks et al. 1979; Manche et al. 1992).

Elbashir et al. (2001a) and Caplen et al. (2001) first demonstrated that small dsRNAs, resembling siRNAs from other systems, induce sequence-specific gene silencing when transiently transfected into mammalian cells. The siRNAs presumably bypass the requirement for Dicer and enter the silencing pathway by incorporation into RISC complexes.

As an alternative strategy, *in vivo* expression constructs for small dsRNA triggers, which resemble endogenously expressed hairpin RNAs, can be delivered to mammalian cells (Brummelkamp et al. 2002a; Paddison et al. 2002b). This approach uses small inverted repeats (19-29 nt) expressed from RNA polymerase III promoter to create short hairpin RNAs (shRNAs), which can then be processed by Dicer and shunted into the RNAi pathway.

1.1.4 Transient and stable RNAi

To knock down target genes in mammalian cells, chemically synthesized 21-nt siRNA duplexes were first used (Caplen et al. 2001; Elbashir et al. 2001a). siRNA can also be transcribed *in vitro* with T7 RNA polymerase, and such siRNA is as effective as chemically synthesized siRNA (Donze and Picard 2002; Yu et al. 2002).
To obtain stable transfection in cells and animals, DNA expression vector-based siRNAs have been developed. In this strategy, a short hairpin RNA (shRNA) is generated in cells under the control of an RNA polymerase III (U6) promoter (Brummelkamp et al. 2002a).

However, the efficiency of siRNA is dependent on the identification of specific target sites because not all sequences are effective in RNA-mediated silencing. To date, the selection of siRNA sequences is still empirical although a few rules can be followed. Generally, several siRNAs derived from different regions of a target mRNA need to be tested in order to achieve an efficient siRNA. Therefore the direct use of

siRNAs for screening of efficiency is warranted. However, a significant disadvantage of this system is that the effects are transient, with phenotypes generated by transfection with such RNAs persisting for 1 week or even less.

In contrary, short hairpin RNAs, generated *in vivo* from expression vectors are potent experimental tools for inducing gene silencing in mammalian somatic cells over long term periods. Not only does this enable the creation of continuous cell lines in which suppression of a target gene is stably maintained by RNAi, but similar strategies may also be useful for the construction of transgenic animals. Thus, short-hairpin-activated gene silencing provides a complement to the use of siRNA in the study of gene function in mammalian cells.

Finally, the ability to encode a constitutive silencing signal may permit the marriage of dsRNA-induced silencing with *in vivo* and *ex vivo* gene delivery methods for therapeutic approaches based on stable RNAi in mammals.

1.1.5 Gene-silencing in therapeutic intervention

The ability of small-interfering RNAs to silence gene expression in somatic mammalian cells has provided researchers with a novel tool to block the expression of disease-causing genes, provided that their mRNA sequences are known. siRNA technology can be applied to a wide range of cancers and other proliferative disorders in which aberrant gene expression occurs. But for the time being, most of the clinical interest lies in applying RNAi in its natural role: as a means of combating pathogenic viruses by disabling their RNA.

Efficient delivery of siRNAs *in vivo* remains, however, a crucial challenge for successful transition from the laboratory to the clinic. Researchers are exploring a variety of ways to solve this problem.

Generally, synthetic siRNAs are delivered to cells in culture via liposome-based transfection reagents. However, only a few of these lipids have been shown to facilitate the uptake of nucleic acids *in vivo* (Safinya 2001) and possible toxic side-effects must be considered.

In chronic diseases, for example, long-term biological effects are desired. Delivery of siRNA can therefore be achieved via a gene therapy approach that relies on the endogenous expression of siRNA from plasmid or viral vectors. Safety issues and the toxicity of these vectors will probably hamper their use in animals or humans (Thomas et al. 2003). Thus, improved viral-based vectors to overcome some

unwanted side-effects are required before the true clinical benefits of RNAi-based therapies can be realized.

And last but not least, another important question mark hanging over the therapeutic use of RNAi is its specificity. Although the actual substrate specificity of individual siRNAs appears to be very high (Elbashir et al. 2001a; Brummelkamp et al. 2002a), recent studies indicate that siRNAs can tolerate single mutations located in the centre of the molecule, and up to four mutations are necessary for complete inactivation (Holen et al. 2002; Jacque et al. 2002; Leirdal and Sioud 2002).

To examine the specificity of siRNAs, global gene expression has been investigated by Semizarov and colleagues using microarray technology (Semizarow et al. 2003). At high concentrations, siRNAs non-specifically induced the expression of a significant number of genes, many of which are known to be involved in apoptosis and stress response. However, reduction of the siRNA concentration eliminated this non-specific response.

Although the recent preclinical studies present compelling data on the utility of siRNA in disease models, we will not know the exact potential that RNAi afford for treating diseases until suitable delivery methods are established to enable the performance of clinical trials. A second key issue is the specificity of siRNAs, and the impact that this might have on their safety in animals and humans.

However this fast-moving field is going on, the goal of this work is to use RNAi as a powerful tool to study the functions of poly(ADP-ribose) polymerases (PARPs).

1.2 Poly(ADP-ribosyl) ation

Poly(ADP-ribosyl) ation is a post-translational modification of proteins. During this process, molecules of ADP-ribose are added successively onto acceptor proteins to form branched polymers. The existence of poly(ADP-ribose) was first reported nearly 40 years ago. Since then, the importance of poly(ADP-ribose) synthesis has been established in many cellular processes.

The synthesis of ADP-ribose requires three distinct enzymatic activities: 1. initiation or mono(ADP-ribosyl) ation of the substrate; 2. elongation of the polymer and; 3. branching of the polymer (Fig. 2).

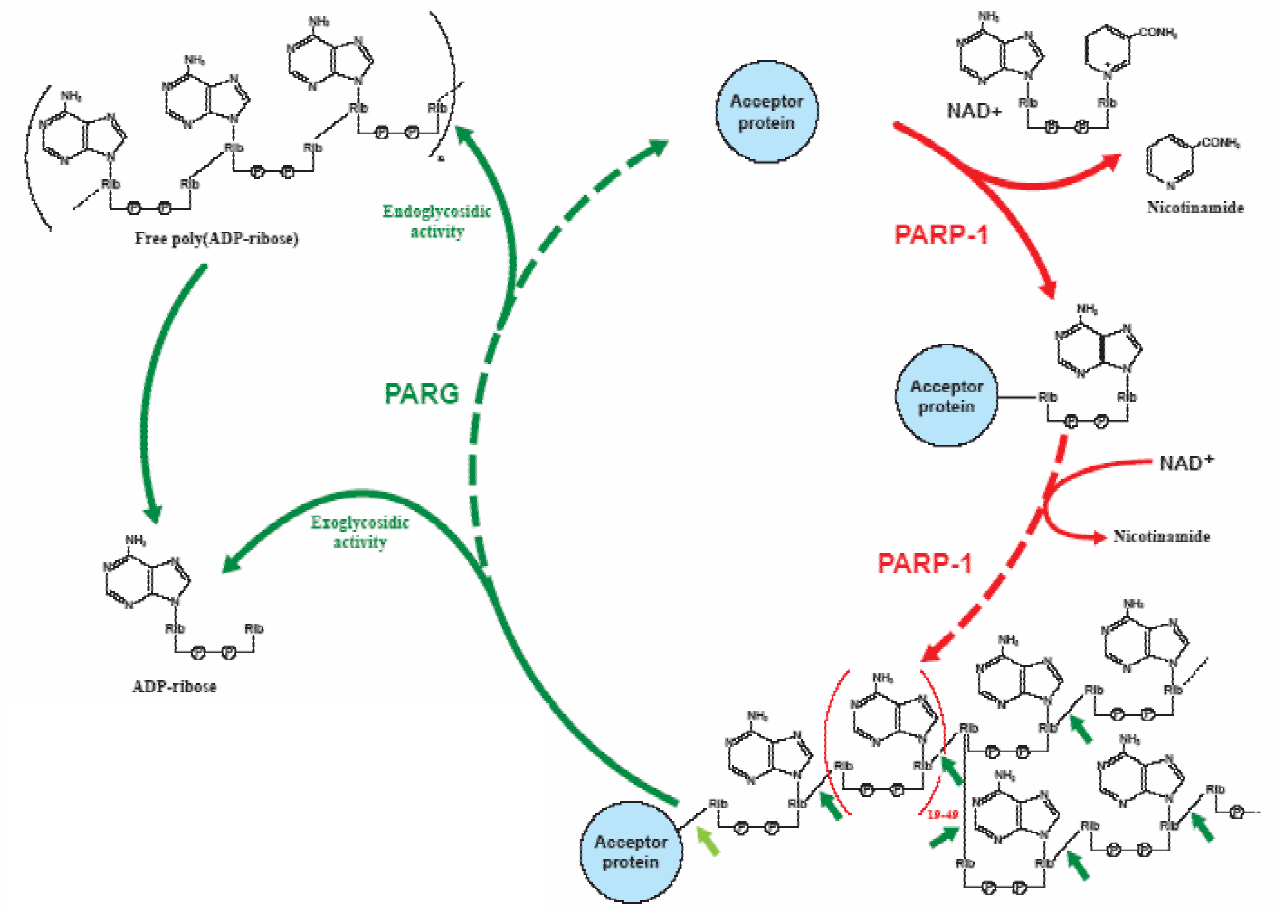


Figure 2: Poly(ADP-ribosyl) ation (Rouleau et al. 2004)

A family of enzymes known as poly(ADP-ribose) polymerases (PARPs) possess all these three activities.

The constitutive levels of polymer are usually very low in unstimulated cells. The majority of the ADP-ribose units found on acceptor proteins in the absence of DNA damage appear as mono- or oligo (ADP-ribose). They are qualitatively different from those synthesized in the presence of DNA damage, and their degradation is far slower than that of polymers synthesized in response to genotoxic agents.

In the presence of DNA strand breaks, the activity of PARP-1, the most abundant member of the PARP family, and the levels of ADP-ribose polymers can be increased several fold (Wielckens et al. 1983; Alvarez-Gonzalez and Althaus 1989; Simonin et al. 1993), while cellular NAD^+ levels are correspondingly reduced (Singh et al. 1985). In living cells, the synthesis of poly(ADP-ribose) is directly proportional to

the number of single- and double-strand breaks present in genomic DNA (Althaus and Richter 1987).

These observations have led to the idea that PARP-1 might act as a "molecular nick sensor"; PARP-1 recognizes and rapidly binds to DNA strand breaks through its zinc fingers and, in turn, its catalytic domain is allosterically activated and starts to synthesize complex branched poly(ADP-ribose) chains. The result is the automodification of PARP-1 itself and, to a lesser extent, the modification of other proteins, including histones.

More than 30 nuclear substrates of PARP-1 have been identified *in vivo* and *in vitro* (Althaus and Richter 1987). However following genotoxic stress, the main acceptor of ADP-ribose *in vivo* is PARP itself (Ogata et al. 1981). Modification of proteins and PARP-1 itself at the site of DNA strand breaks favours the repair process and acts as a strong signal for the recruitment of DNA damage-signalling molecules (Althaus 1992; Althaus et al 1999).

Reversibility: PARG and the catabolism of PAR

Like other covalent protein modifications (e.g. phosphorylation, acetylation), poly(ADP-ribosyl) ation is reversible (Amé et al. 2000; D'Amours et al. 1999). The efficient degradation of poly(ADP-ribose) requires three different enzymatic activities, which are carried out by two distinct enzymes: poly(ADP-ribose) glycohydrolase (PARG) and ADP-ribosyl protein lyase. The existence of PARG was first demonstrated by Miwa and Sugimura (1971) and by Ueda et al. (1972). Human and bovine PARG cDNA have been cloned, and were shown to encode proteins of 111 kDa (Lin et al. 1997). PARG possesses exoglycosidase (Miwa et al. 1974) and endoglycosidase (Ikejima and Gill 1988) activity. These activities are responsible for the hydrolysis of glycosidic bonds between ADP-ribose units located at the extremity and within the polymer, respectively.

ADP-ribosyl protein lyase is the enzyme responsible for the hydrolysis of the most proximal unit of ADP-ribose on the protein acceptor.

In vivo, the steady-state cellular levels of poly(ADP-ribosyl)ation are determined by the opposing actions of PARPs and PARG (Davidovic et al. 2001). PARG activity appears to increase proportionally with polymer size, allowing PARG to counteract the actions of PARP more effectively for long polymers.

Once freed from the polymer, ADP-ribose units are catabolized to AMP and ribose 5'-phosphate by (ADP-ribose) pyrophosphatases (Miro et al. 1989; Fernandez et al. 1996).

1.2.1 PARP-1 structure

PARP-1 (113 kDa) is an abundant nuclear protein found in all eukaryotes except yeast.

In the late 1980's the cloning of the human PARP gene allowed its localization on chromosome 1 and established that it consists of 23 exons spanning approximately 42 kb of DNA (Cherney et al. 1987). The same organization was found for the murine gene, albeit spanning only 33 kb (Berghammer et al. 1992). The gene codes for a protein of 1014 amino acids which has been isolated in high levels from testis, thymus and spleen, while lower levels were found in liver, kidney and heart. On the subcellular level, PARP is present primarily in the nucleus, albeit with non-homogeneous distribution.

The primary structure of PARP-1 consists of four main distinct regions:

1. A N-terminal DNA-binding domain (DBD) bearing two zinc fingers (FI, FII) acting both as a molecular nick sensor and as an interface for interaction with protein partners
2. A bipartite nuclear location signal interrupted by a caspase-3 cleavage site, at which PARP-1 is cleaved during apoptosis.
3. A central automodification domain containing a BRCT motif involved in protein-protein interactions. This region also contains auto-poly(ADP-ribosyl)ation sites implicated in the negative regulation of PARP-DNA interactions.
4. A C-terminal catalytic domain containing a donor site (NAD^+binding) and an acceptor site (polymer binding). This domain, highly conserved during evolution, is responsible for poly(ADP-ribose) synthesis.

The primary structure of PARP-1 presents high interspecies homology, with human and mouse variants sharing 92% homology. The C-terminal part, and particularly residues 859 to 908, represents the most highly conserved region among PARPs (Fig. 3). This amino acid stretch has therefore been termed the "PARP signature sequence".

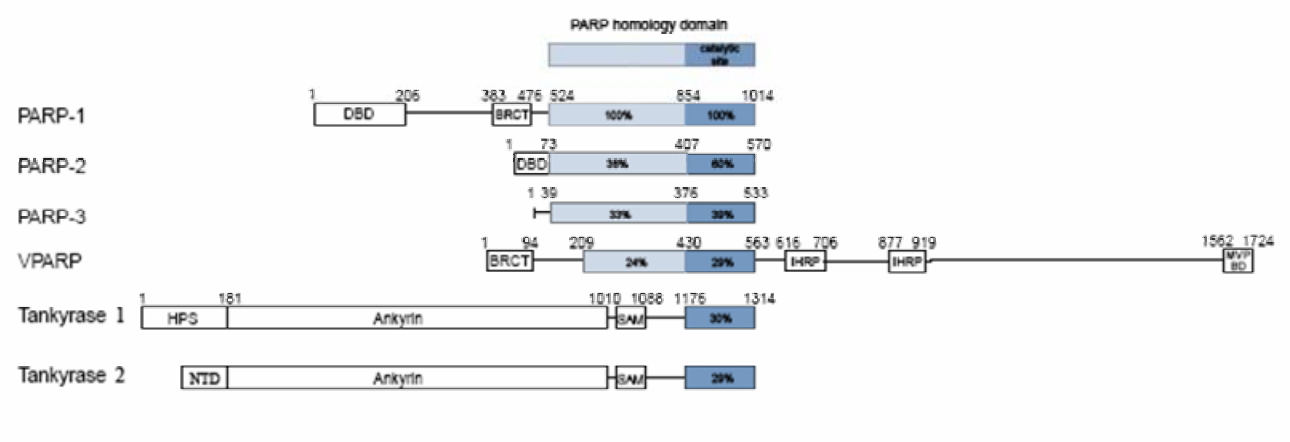


Figure 3: Schematic structure of six different members of the PARP family. Percentages indicate the amino acid identities to PARP-1 (Modified from Smith, 2001).

1.2.2 Biological roles of PARP-1

PARP-1 and DNA transcription

Poly(ADP-ribosyl)ation participates in the regulation of transcriptional activity. The available data suggest that PARP-1's activity in transcriptional regulation occurs by at least two mechanisms that are not mutually exclusive: 1. modifying histones to alter chromatin structure and 2. functioning as part of enhancer/promoter binding complexes in conjunction with other DNA binding factors and coactivators.

Poly(ADP-ribosyl) ation of histones contributes to the transcription-promoting effect of PARP-1. In fact poly(ADP-ribose) confers negative charges to histones, leading to electrostatic repulsion between histones and DNA. Thus, poly(ADP-ribosyl) ation can loosen the chromatin structure and can thereby make genes more accessible for the transcriptional machinery (D'Amour et al. 1999).

In addition, PARP-1 activity at enhancers and promoters is mediated in large part by functional interactions between PARP-1 and various non-histone proteins, many of which are DNA binding transcription factors, including NF-κB (Hassa and Hottiger 1999), p53 (Whitacre et al. 1995), B-MYB (Cervellera and Sala 2000), and nuclear receptors (Miyamoto et al. 1999), to name some of them.

PARP-1 modulates gene expression in both a positive and a negative fashion, with the final effects depending on the cell type, the gene and the transcription factor involved (Ziegler and Oei 2001).

PARP-1 and the maintenance of genomic stability

PARP-1 has been implicated in DNA-repair and maintenance of genomic integrity (de Murcia and Menissier de Murcia 1994; Schreiber et al. 1995). This "guardian angel" function is indicated by delayed DNA base-excision repair and by a high frequency of sister chromatid exchange (SCE) in PARP-1-deficient cells exposed to ionizing radiation or treated with alkylating agents (de Murcia et al. 1997). For the PARP knockout studies, the frequency of SCE was 2-5 fold higher either before or after DNA damage. In addition to an increased SCE frequency, PARP-1 knockout cells contain increased levels of micronuclei after DNA damage (Wang et al. 1997). PARP-1 null mutation in mice causes also high levels of aneuploidy, chromosomal fragmentation and fusion as well as chromosome loss and gain (d`Adda di Fagagna et al. 1999; Tong et al. 2001; Simbulan-Rosenthal et al. 1999). These data together demonstrate that PARP-1 acts as a genome guardian molecule.

Poly(ADP-ribosyl) ation, NAD^+ metabolism and cell death

Paradoxically, despite the beneficial effect in the maintenance of genomic stability, PARP-1 can induce cell death through NAD^+ depletion.
It is well established that excessive DNA damage causes PARP-1 hyperactivation, which in turn depletes NAD^+ pools within only a few minutes (D`Amour et al. 1999; Shall and de Murcia 2000). Consequently, the main NAD^+-dependent metabolic pathways such as glycolysis and mitrochondrial respiration are impaired, leading to reduced ATP production and cellular dysfunction. Moreover, under these conditions phosphoribosyl pyrophosphate synthetase and nicotinamide mononucleotide adenylyl transferase consume ATP in effort to resynthesize NAD^+, worsening the energetic shortage and contributing to the generation of a lethal, futile cycle. This pathway is identified as a causal event of necrotic cell death.

Role of poly(ADP-ribosyl) ation in disease

The use of PARP-1 knockout mice and selective PARP inhibitors in various models of disease has provided interesting insights at what might be the role of the

enzyme's activation. When overactivation of PARP-1, which leads to energy-depletion by consumption of NAD^+ levels, takes place on the scale of an organ (e.g., during the ischemia and reperfusion of the brain or the heart), necrosis on a large scale leads to the loss of organ function and ultimately to death.
Therefore PARP-1 has been implicated in the pathogenesis of several diseases that are characterized by excessive PARP-1 activity, like stroke, myocardial ischemia, diabetes, diabetes-associated cardiovascular dysfunction, shock, traumatic central nervous system injury, arthritis, colitis, allergic encephalomyelitis, and various other forms of inflammation.

Accordingly, the inhibition of PARP-1 activity is a valid therapeutic strategy in a wide number of experimental disorders and has been a field of intense investigation for both basic scientists and pharmaceutical companies.

1.2.3 The PARP family

The first hint of the presence of more than one PARP enzyme existing in cells came from the observation of poly(ADP-ribose) synthesis in mouse embryonic fibroblasts derived from PARP-1 knockout mice (Shieh et al. 1998). Since then numerous reports have appeared describing new members of a still expanding family. At the time of this writing, the family consists of 18 PARP proteins; 6 of them are more or less well characterized.

PARP-2

In 1999, Amé et al. described the isolation and characterization of a new member of the family, named PARP-2 (Amé et al. 1999).

The PARP-2 gene maps to chromosome 14C1 and 14q11.2 in mouse and human, which are distinct from PARP-1 loci, supporting the conclusion that PARP-2 is coded by a different gene.

PARP-2 bears a strong resemblance to PARP-1. An alignment of the sequences showed that the carboxy-terminal region of PARP-2 shared 43% identity with PARP-1. This sequence contains the PARP signature, i.e. the ADP-ribose donor site and the crucial residues of the acceptor site.

PARP-2 (like PARP-1) is a nuclear protein that binds to and is activated by DNA strand breaks (Amé et al. 1999). Interestingly the DNA-binding domain of

PARP-2 is distinct from PARP-1 and could indicate different substrate specificities and possibly different functional roles for these two proteins. Nonetheless, it is likely that PARP-2 contributes to the residual poly(ADP-ribose) synthesis observed in PARP-$1^{-/-}$ cells after treatment with DNA-damaging agents.

PARP-3

PARP-3 is a 533 amino acid nuclear protein with a mass of 60.1 kDa (Johannsson et al. 1999). It consists of a unique N-terminal domain of 39 amino acids, followed by the PARP homology domain. Recombinant hPARP-3 localizes *in vitro* during the entire cell cycle to the centrosome, the microtubule organizing centre of animal cells, and resides preferentially in the daughter centriole. hPARP-3 has been shown to negatively influence the G1/S cell cycle progression without interfering with centrosome duplication (Augustin et al. 2003). The presence of both PARP-1 and PARP-3 at the centrosome may link the DNA damage surveillance network to the mitotic fidelity checkpoint but future studies are necessary to reveal the specific functions of PARP-3.

VPARP

VPARP or PARP-4 was identified as a component of the vault complex. The vault complexes are with a mass of 13 MDa the largest ribonucleoprotein particles described to date. Vaults are barrel-shaped cytoplasmic particles that are composed of a major vault protein (MVP), two minor vault proteins [telomerase-associated protein 1 (TEP1), vault poly(ADP-ribose) polymerase (VPARP)] and small untranslated RNA molecules.

The function of these unusual particles is unknown; however, their subcellular localization and distinct morphology point to a role for vaults in intracellular, particularly nucleo-cytoplasmic, transport (Abbondanza et al. 1998; Li et al. 1999).
Unique features of VPARP are a BRCA1 C-terminus (BRCT) domain (aa1-94) and an inter-α-inhibitor domain (aa616-1195); both domains may be involved in protein-protein interactions. The C-terminus of VPARP (aa1562-1724) has been shown to associate with the N-terminal part of MVP (Kickhoefer et al. 1999; van Zon et al. 2002).

Immunofluorescence and biochemical fractionation studies clearly indicate that not all VPARP is bound to vaults. VPARP is also present in the nuclear matrix and in cytoplasmic clusters (VPARP-rods) (Kickhoefer et al. 1999; Schroeijers et al. 2000). Vault poly (ADP-ribose) polymerase exhibits a poly (ADP-ribose) polymerase activity and can poly (ADP-ribosylate) MVP and to a lesser extent, itself (Kickhoefer et al. 1999). Whether there are other substrates of VPARP is presently unknown. It is also not yet clear whether VPARP fulfils separate functions – unrelated to vault function – in its non-vault associated form.

Tankyrase 1

Tankyrase, also known as PARP-5, is a 142 kDa protein that has been localized to the human telomers (Smith et al. 1998). Telomeres, which are essential for chromosome maintenance and stability, are maintained by telomerase, a specialized reverse transcriptase.

Tankyrase was initially identified through its interaction with TRF1, a negative regulator of telomere length (van Steensel and de Lange 1997). Tankyrase was found to poly(ADP-ribosyl) ate itself and TRF1 and this modification inhibited the binding of TRF1 to telomeric RNA (Smith et al. 1998).

Overexpression of tankyrase affects TRF1 at telomeres and promotes telomere elongation in human cells (Smith and de Lange 2000), indicating that tankyrase can function as a telomere-length regulator.

Tankyrase 2

In 2000, studies revealed a tankyrase homolog, termed tankyrase 2 (Chi and Lodish 2000), which exhibits more than 80% identity with tankyrase. The localization and function of this tankyrase homolog is not yet known.

1.3 Aim of this work

PARP-1 and PARP-2 are both involved in the response to DNA damage. However a specific female embryonic lethality has been identified in PARP-$1^{+/-}$ PARP-$2^{-/-}$ mice associated with X-chromosome instability, suggesting that the two enzymes have both non-redundant and overlapping functions in the maintenance of genomic stability.

The fact that there exist more than one PARP protein in the cells and that PARP-1$^{-/-}$ PARP-2$^{-/-}$ mice are not viable makes it difficult to characterize the distinctive functions of PARP-1 and PARP-2. Based on this knowledge, the aim of this work was to set up the best conditions for a knockdown of PARP-1 by RNA interference using either *in vitro* transcribed siRNA molecules or a plasmid vector producing the siRNA molecules within the cells. The knockdown of PARP-1 expression in mammalian cells will be the basis for further work to elucidate the distinct functions of the enzymes PARP-1 and PARP-2.

2. MATERIALS

2.1 Cell culture

Cell culture medium, Dulbecco`s Modified Eagle`s Medium (DMEM), fetal bovine serum, antibiotics (as Penicillin-Streptomycin mixture) and trypsin-EDTA were all purchased from GibcoBRL; Invitrogen, Carlsbad, CA, USA).

2.2 Chemicals, enzymes and plasmids

GFP-p53 plasmid was purchased from Clontech, pSilencer 1.0-U6 siRNA Expression vector was purchased from Ambion.

H_2O_2 (Hydrogenperoxide) was purchased from Fluka as a 10,2 M stock solution and stored at 4 °C in the dark.

Agarose, molecular biology grade was from SIGMA.

The following transfection reagents were used:

ExGen500 (Fermentas)

Jet PEI™ (Qbiogene)

Lipofectamin™ (Invitrogen)

Polyfect® (QIAGEN)

Transferrinfection (Alexis)

Metafectene (Biontex)

2.3 Kits

The following kits were purchased from QUIAGEN:

QIAquick Gel Extraction Kit

QIAprep Spin Miniprep Kit

QIAGEN EndoFree™ Plasmid Purification Kit

The Silencer™ siRNA Construction Kit was from Ambion.

The SuperSignal®West Femto Maximum Sensitivity Substrate kit was obtained from PIERCE.

2.4 Antibodies

2.4.1 Primary antibodies

Monoclonal anti-PARP-1 antibody (C2-10) was purchased from Alexis Biochemicals, CA, USA and was used at 1: 5000 dilution.

Monoclonal anti-GAPDH antibody was obtained from Ambion, Texas, USA. The working dilution was 1:40000.

Anti-green fluorescent protein antibody (anti-GFP) (rabbit IgG fraction) was obtained from Molecular Probes (2 mg/ml). Working dilution was 1:200.

2.4.2 Secondary antibodies

Goat anti-mouse (Fab specific) and goat anti-rabbit (whole molecule) antibodies were purchased from Sigma, Saint Louis, Missouri, USA. Both antibodies were peroxidase-conjugated and were used for development with the enhanced chemiluminescence (ECL) technique.

3. METHODS

3.1 Cell culture

Mouse embryonic fibroblasts (MEFs) were derived from PARP-$1^{+/+}$ and PARP-$1^{-/-}$ mice and established in culture according to 3T3 protocol (Wang et al. 1995).

Cells were cultured at 37 °C in saturated humid atmosphere under 5 % CO_2 in Dulbecco`s Modified Eagle`s Medium (DMEM) containing 4500 mg/l glucose, supplemented with 10% fetal bovine serum, 100000 units/l penicillin and 100 mg/l streptomycin (complete DMEM).

Cell stocks were stored frozen in liquid nitrogen. To start the cultures, cells were thawed at 37°C, transferred into sterile tubes, diluted with cold complete medium and collected by centrifugation (5 minutes, 1000 rpm, 4°C; Eppendorf centrifuge 5810 R, Hamburg, Germany). Then cells were resuspended into prewarmed complete medium and transferred into 75 cm^2 flasks. Medium was changed after 6-12 hours.

Subculturing was done when the cultures reached confluence (every second to third day), at a split ratio 1:10. The cells were washed twice with prewarmed sterile PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4), trypsinized (0.5 % Trypsin in PBS), collected in 15 ml cold medium and centrifuged at 1000 rpm at 4°C for 5 min in an Eppendorf centrifuge 5810 R, Hamburg, Germany. Cells were resuspended in warm complete DMEM and transferred to the appropriate culture vessels. The medium was changed after 12 hours.

3.2 Cell Treatments

3.2.1 Transfection of pSilencer™1.0-U6

The expression vector pSilencer™1.0-U6 (pSU6) was purchased from Ambion and modified to contain specific insert sequences as reported in “Results”. Transfection was done by either chemical or physical (electroporation) methods.

In separate experiments, chemical transfection was achieved by the use of the following reagents: Lipofectamin™ (Invitrogen), Polyfect® (QIAGEN), Metafectene

(Biontex), Ex Gen 500 (Fermentas), Jet PEI™ (Qbiogene), and Transferrinfection (Alexis).

All transfections were performed in the conditions recommended by the manufacturers including optimization of vector : transfection reagent ratios.

Alternatively, cells were collected by trypsinization into Falcon Tubes and resuspended in incomplete DMEM at 4 x 10^6 cells/ml. Four hundred μl of suspension were transferred into each electroporation cuvette (BioRad) and pSU6 expression vector (1-5 μg/μl) was added to cells. The suspension was mixed by flicking the bottom of the cuvette. Electroporation was performed using the BioRad electroporator GENE PULSER II at 950 μF capacitance and 0.25 kV. After electroporation the cells were seeded in 2 ml complete DMEM in 6 well plates.

3.2.2 siRNA transfection

Specific small interfering RNAs (siRNAs) were synthesized *in vitro* as described in section 2.9.

Prior to transfection, 2 x 10^4 cells were seeded in 24 well plates until they reached 50 % confluence (about 24 hours). siPORT Amine (Ambion) was used as transfection reagent in conditions recommended by the manufacturer. Briefly, siPORT Amine was mixed (1:12; v:v) with OPTI-MEM I medium and incubated for 10-30 minutes at room temperature. siRNA (20 μM) was added to diluted siPORT Amine transfection agent to a final concentration of 0.4 μM. The solution was mixed gently by pipetting and incubated at room temperature for 15-20 minutes. Fifty μl of this mixture (transfection agent/siRNA complex) were added dropwise to the 24 well plate containing 200 μl OPTI-MEM I/well and the dish was rocked to distribute the solution. The final concentration of siRNA in the plate wells was 80 nM. Cells were transferred to the incubator, and after 4-8 hours 1 ml of prewarmed complete DMEM was added to each well, to maximize cell growth and prevent potential cytotoxicity.

3.2.3 Induction of DNA damage

Oxidative DNA damage was induced by treatment with 100 μM H_2O_2 in incomplete medium at 37 °C. At the end of the treatment period, pictures were taken by a CCD Color Camera (KAPPA) connected to an Inverted Microscope DIAPHOT-TMD (Nikon) and a Trinitron Color Video Monitor (Sony).

3.3 Preparation of whole cell lysates

Cells in 24 well culture plates were placed on ice and washed twice with ice cold PBS. 200 µl of SDS sample buffer (Laemmli 1970) were added to each well and cells were scraped with a rubber policeman and collected into sterile Eppendorf tubes. Lysed cells were sonicated (5 cycles, 5 seconds, 37 % power; Bandelin Sonopuls, Berlin, Germany) and after heating at 95 °C for 2 minutes, insoluble material was discarded by centrifugation at 10000 rpm for 10 minutes (microfuge 5415 R, Eppendorf, Hamburg, Germany). Supernatants were stored at –80°C until use.

3.4 Protein quantification

Protein concentrations were determined by the method of Lowry et al. (1951). Briefly, tubes containing either increasing amounts of BSA (from 1 mg/ml stock solution) or samples in lysis extraction buffer were prepared in duplicates. One ml of reaction mix (0.01% $CuSO_4$ (w/v), 0.02% sodium potassium tartrate ($C_4H_4NaKO_6 \bullet 4H_2O$, w/v), 0.2 M NaOH and 4% Na_2CO_3) was added and the tubes were incubated for 10 minutes at room temperature. After incubation, 100 µl of 50% (v/v) Folin and Ciocalteu`s phenol reagent were added. The tubes were immediately vortexed and incubated for another 10 minutes. Finally, absorbance at 750 nm was determined on an Ultraspec Plus spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK). Protein concentration was calculated by extrapolation on a standard curve plotted with data relative to BSA.

3.5 Electrophoretic analyses

3.5.1 Agarose gel electrophoresis

Electrophoresis was performed on 1 % agarose gels containing 0.5 µg/ml ethidium bromide. The gels were run horizontally in 1 x TBE buffer (89 mM TRIS, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 80 V for 1.5 hours. Results were visualized under UV light.

3.5.2 SDS-PAGE

Proteins were separated on polyacylamide gels by standard SDS-PAGE technique (Laemmli 1970), using a Bio-Rad vertical electrophoresis unit. Ten % polyacrylamide gels were prepared, from a stock solution containing 29.2% (w/v) acrylamide and 0.8% (w/v) N,N-methylen-bisacrylamide.
Electrophoresis was carried out at 125 V for 90 minutes in 25 mM Tris, 125 mM glycin, pH 8.3, containing 0.1% (w/v) SDS.

3.6 Western blot analysis

Following separation by SDS-PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (0.2 µm pore size, Bio-Rad, Hercules, CA, USA) using the Bio-Rad tank transfer system. The PVDF membranes were pre-soaked in methanol and equilibrated in transfer buffer (12 mM TRIS, 96 mM Glycine, 20% (v/v) Methanol). Transfer was accomplished at 125 V for 1.5 hours at room temperature. After transfer, the membrane was washed 3 times with TBST (10 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 7.4). To block unspecific binding sites, membranes were incubated for 18 hours at 4 °C in TBST containing 5% (w/v) non-fat dry milk (TBSTM). Primary antibodies were diluted to desired concentrations (see section: 3.4 Antibodies) in 2.5% TBSTM and incubation was carried out in glass tanks for 60 minutes, at room temperature, on an orbital shaker.

The membranes were subsequently washed 3 times for 10 minutes in 2.5% TBSTM, and then incubated for 60 minutes with appropriate peroxidase conjugated secondary antibodies diluted 1:10000 (anti-mouse IgG) or 1:15000 (anti-rabbit IgG), in 2.5% TBSTM. Finally the membranes were rinsed twice in 2.5% TBSTM and twice in TBST.

Blots were developed by the technique of enhanced chemiluminescence (SuperSignal®West Femto Maximum Sensitivity Substrate kit from PIERCE). Immunodetected bands were quantified on a Computing Densitometer 325 (Molecular Dynamics, now part of Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) using ImageQuant 3.3 (Molecular Dynamics) running on Windows 3.1 (Microsoft, Redmond, WA, USA). Data were normalized to GAPDH content in all analyses and expressed as ratio to the corresponding values in control cells.

3.7 Staining of protein blots

Protein blotted on PVDF membranes were stained with Coomassie Blue. The membranes were equilibrated 2 minutes in 50% methanol/10% (v/v) acetic acid solution and then incubated with Coomassie staining solution (0.1% (w/v) Coomassie Blue R in 45% methanol/10% (v/v) acetic acid) for 1 minute with gentle agitation. After washing with 50% methanol/10% (v/v) acetic acid solution and incubation in H_2O for 10 minutes, the membranes were finally air-dried and stored in plastic foil.

3.8 Indirect immunofluorescence

Cells grown on coverslips were rinsed twice in PBS and then fixed at -20 °C in ice-cold acetone methanol (1:1 (v/v)) for 20 minutes. After rehydration in PBS, cells were incubated for 1 hour in blocking solution (10 % (v/v) FBS, 0.01 % (v/v) Triton X-100 in PBS, PBS-ST), followed by incubation with primary antibodies in PBS-ST, with gentle agitation. After 1 hour, coverslips were rinsed 3 times with PBS, and incubated for additional 1 hour with secondary antibodies conjugated with fluorescent marker (dilution 1:100). After 3 PBS washes, coverslips were incubated for 1 minute in DAPI (4',6-Diamidino-2-phenylindole) (10 μg/ml) and finally washed 3 times in PBS. Coverslips were air-dried, mounted on ethanol cleaned slides with Mowiol (Calbiochem, San Diego, USA) and analyzed under fluorescence microscope.

3.9 RNAi constructs

3.9.1 siRNA Oligonucleotide templates design

In principle the selection of potential target sequences follows few rules that are applicable to both oligonucleotide templates for siRNA transcription *in vitro* and to insert sequences for an expression vector.

Target sequences for siRNA were identified by first scanning target genes for AA sequences. The AA and downstream 19 nucleotides were recorded and compared to the mouse genome database to eliminate any sequences with significant homology to other genes. Oligonucleotides were designed to contain the selected gene-specific sequences in addition to sequences required for either *in vitro* transcription or cloning into expression vectors (see “Results”, paragraphs 4.1 and 4.2) and were purchased from Microsynth, Balgach, Schweiz. A pending patent application precludes sequence specifications of the siRNAs used in these studies.

3.9.2 Construction of siRNA expression vector

siRNA Insert Preparation

Forward and reverse oligonucleotides for the siRNA insert were dissolved in 10 mM TRIS-HCl (pH 8.0) to a final concentration of 1 µg/µl. Oligonucleotides were annealed in HEPES-buffer (30 mM HEPES-KOH, pH 7.4; 100 mM K-acetate; 2 mM Mg-acetate) by heating to 90 °C for 3 minutes followed by incubation at 37 °C for 1 hour.

Cloning of the siRNA sequences into pSilencer^{TM} 1.0-U6

The pSilencer^{TM}1.0-U6 Vector was digested with *Eco* R I and *Apa* I restriction enzymes (BioLabs, New England) in succession. In a 50 µl reaction mixture 10 µg of pSU6 were incubated with NEBuffer *Eco* R I (50 mM NaCl; 100 mM Tris-HCl; 10 mM $MgCl_2$; 0.025 % Triton X-100 (pH 7.5)) and 20 units *Eco* R I at 37 °C for 1 hour. The reaction was stopped by heat inactivation of the enzyme at 65 °C for 20 minutes and the digested plasmid was analyzed by 1% agarose gel electrophoresis. Linearized vector was purified by extraction from agarose gel (QIAquick Gel Extraction Kit).

The linearized vector was incubated in NEBuffer 4 (50 mM potassium acetate; 20 mM Tris-acetate; 10 mM magnesium acetate; 1 mM dithiothreitol (pH 7.9)), containing 0.1 mg/ml BSA and 20 units *Apa* I at 25 °C for 1 hour, in 50 µl final volume. Reaction was stopped by heat inactivation of the enzyme at 65 °C for 20 minutes followed by analysis on 1 % agarose gel of the product. Linearized vector was extracted from agarose gel, and purified as described above. The purified linear plasmid was quantified by measuring the absorbance at 260 nm by an Ultraspec Plus spectrophotometer (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) and diluted to a final concentration of 0.5 µg/µl.

Finally, 500 ng *Eco* R I/*Apa* I linearized expression vector were mixed with 16 ng double-stranded siRNA insert and incubated with 1 unit T4 DNA Ligase in the appropriate buffer (Ambion), at room temperature, overnight.

Amplification of pSilencer^{TM} 1.0-U6 siRNA Expression Vector and selection of positive clones

Five µl of ligation reaction mixture or 10 ng of pSU6 empty vector were used to transform competent *E.coli* DH5α cells (100 µl) by heat shock. Transformants were

plated onto Luria-Bertani Medium (LB) agar plates containing 100 mg/l ampicillin. After 16 hours, single colonies were inoculated in LB medium containing 100 mg/l ampicillin and grown at 37 °C for 24 hours. Plasmids were isolated by spin column chromatography using the QIAprep Spin Miniprep Kit. Plasmid concentration was determined by measuring the absorbance at 260 nm. Purified plasmids (2 µg) were digested with 20 units *Hind* III (BioLabs, New England) in NEBuffer 2 (50 mM NaCl, 10 mM TRIS-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, (pH 7.9)) in a 50 µl reaction mixture, at 37 °C for 1 hour to identify the plasmids containing the siRNA inserts, digestion products were analyzed by 1% agarose gel. Positive plasmids were amplified in competent DH5α cells, as described before, and purified using the QIAGEN EndoFree™ Plasmid Purification Kit. Aliquots of transformed bacteria were frozen as glycerol stock solutions at -80 °C.

3.9.3 siRNA synthesis by *in vitro* transcription

siRNAs were synthesized using the Silencer™ siRNA Construction Kit (Ambion). In separate reactions, dry supplied sense and antisense 29-mer DNA oligonucleotide templates with 21 nt encoding the siRNA and 8 nt complementary to the T7 Promoter Primer were dissolved in nuclease-free TE-Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), to have a final concentration of 100 µM. Each DNA oligonucleotide template was hybridized to the T7 promoter primer in “DNA Hyb Buffer” provided with the kit, by heating to 70 °C for 5 minutes followed by 5 minutes cooling at room temperature. The 3’ ends of the hybridized oligonucleotides were extended using a DNA polymerizing reaction, as described in the kit protocol. The resulting ds oligonucleotide templates were used in an *in vitro* transcription reaction to synthesize sense and antisense siRNAs. The two RNA products were hybridized by combining by overnight incubation at 37 °C. To remove 5’ overhanging leader sequences and to eliminate DNA templates, the dsRNA hybridization mixture was subjected to digestion with single-strand specific RNase and DNase by incubation at 37 °C for 2 hours in the appropriate buffer.

siRNA was separated from nucleotides, enzymes, short oligomers and salts present in the reaction mixture by column chromatography (Silencer™ siRNA Construction Kit, Ambion). siRNA was eluted from column into 100 µl of nuclease-free water; the concentration was measured by reading the absorbance at 260 nm

and adjusted to 20 µM. The quality of siRNAs was analysed by 1% agarose gel electroporesis.

An outline of siRNA synthesis procedure is given in Fig. 4.

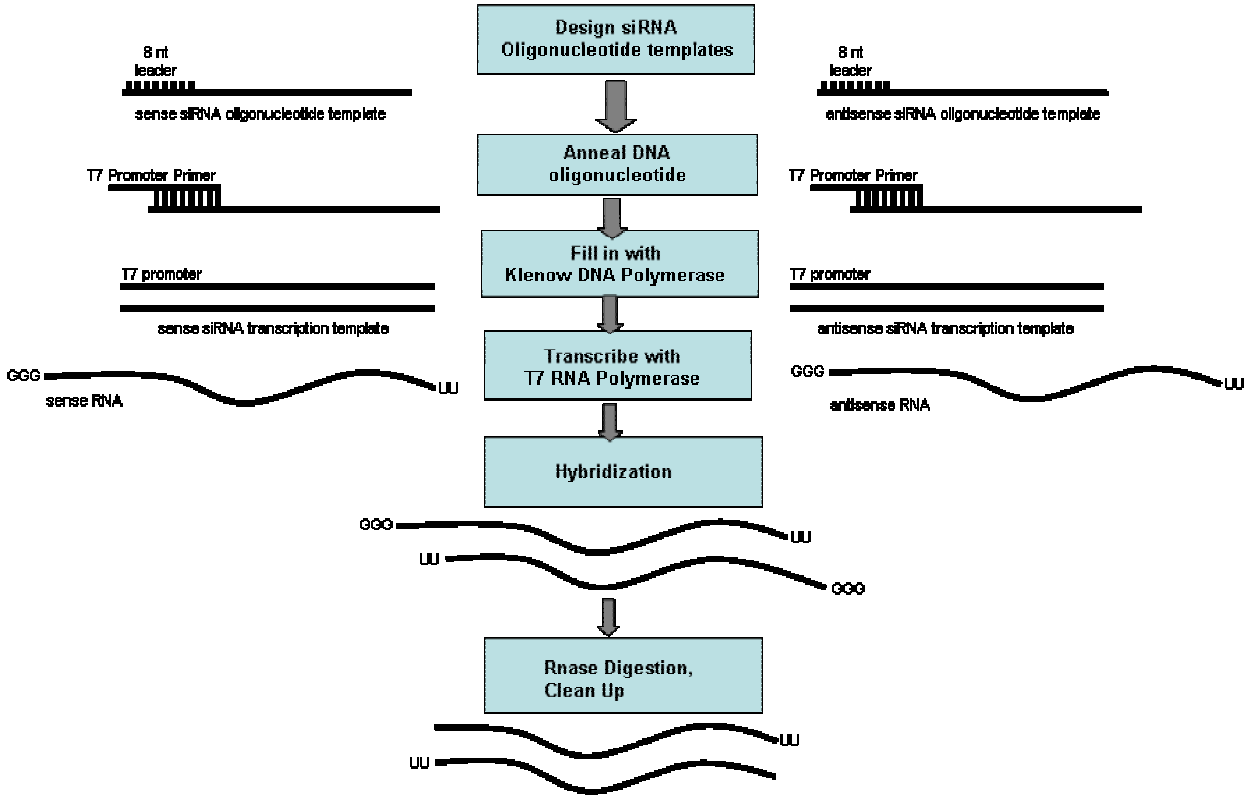


Figure 4: Silencer siRNA Construction (Ambion)

4. RESULTS

4.1 RNAi by the siRNA expression vector pSilencer™ 1.0-U6

The pSilencer 1.0-U6 (pSU6) siRNA Expression Vector has been designed for plasmid-based siRNA experiments. It is a 3.3 Kb vector, containing sequence elements for cloning and bacterial replication, i.e. f1 origin, ColE1 origin, Ampicillin resistance gene, and multiple cloning site (Fig. 5). A U6 RNA Polymerase III (Pol III) promoter (-315 to +1) is cloned into the *Kpn* I and *Apa* I sites to generate small RNA transcripts. The U6 Pol III promoter was chosen because Pol III has well-defined initiation and stop sites from its promoter to a string of 4-5 uridines.

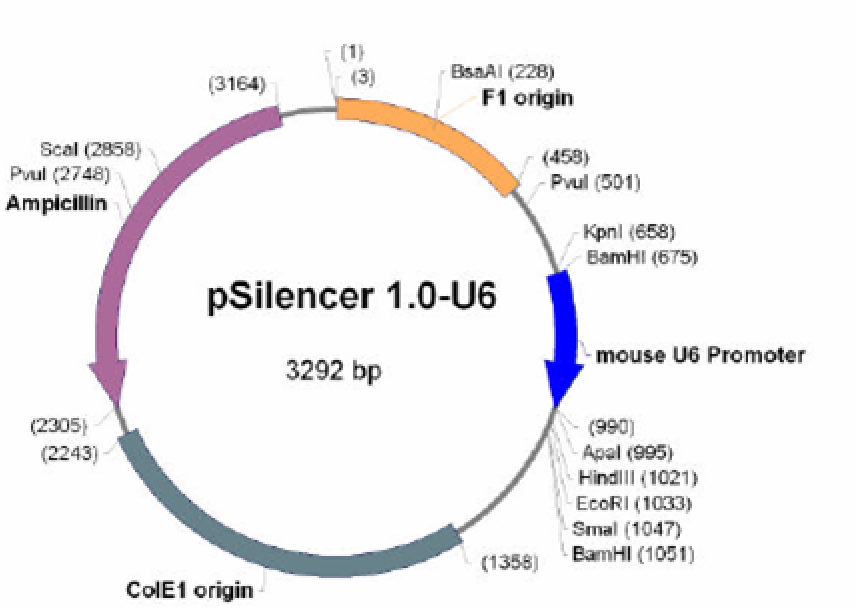


Figure 5: pSilencer™1.0-U6 siRNA Expression Vector (Ambion)

In general, after selection of 19-nucleotide siRNA sequences, downstream of AA dinucleotides (see Methods 2.9.1) on the mRNA of the gene of interest, two DNA oligonucleotides, ~55 nt in size, are designed as insert sequences (Fig. 6).

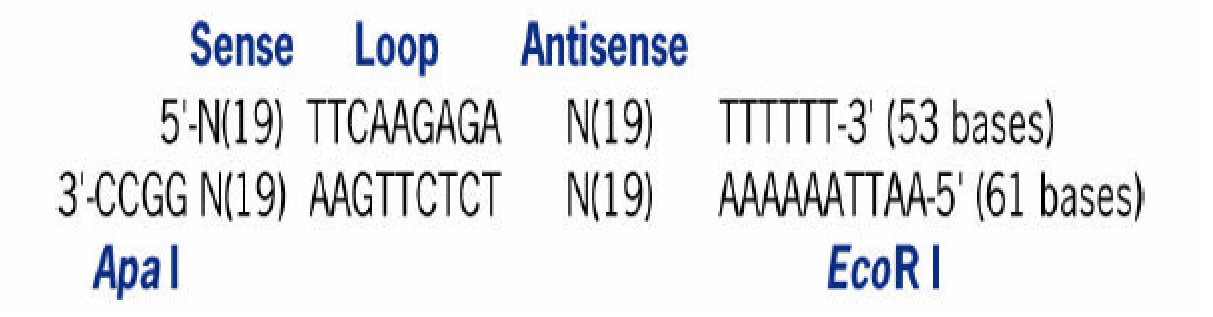


Figure 6: Schematic representation of a siRNA insert

In the forward oligonucleotide, the 19-nucleotide sense siRNA sequence is linked to the reverse complementary antisense siRNA sequence by a 9-nucleotide spacer (TTCAAGAGA). 5-6 Ts are added to the 3' end of the oligonucleotides. In the reverse oligonucleotide, 4-nucleotide overhangs to the *Eco*R I (AATT) and *Apa* I (GGCC) restriction sites are added to the 5' and 3' end of the 54 nt sequence complementary to the forward oligonucleotide, respectively. The resulting RNA transcript is likely to fold back and form a stem-loop structure comprising 19 bp stem and 9 nt loop with 2-3 Us at the 3' end (Fig. 7).

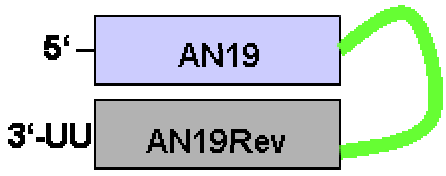


Figure 7: Haipin structure of the RNA transcript

4.1.1 Insertion of siRNA sequences into pSU6

The pSU6 expression vector was first linearized with the restriction enzyme *Apa* I as described under "Materials and Methods". The completion of the digestion reaction was controlled by agarose gel electrophoresis. As shown in Fig. 8 A, the fast migrating band, corresponding to supercoiled pSU6 (lane 1), was converted to the

slower migrating linear form (lane 2), at the end of the incubation time. In a second step, the linearized vector was digested with *Eco* R I and purified. The final digestion product migrated as a single band on agarose gel (Fig. 8 B).

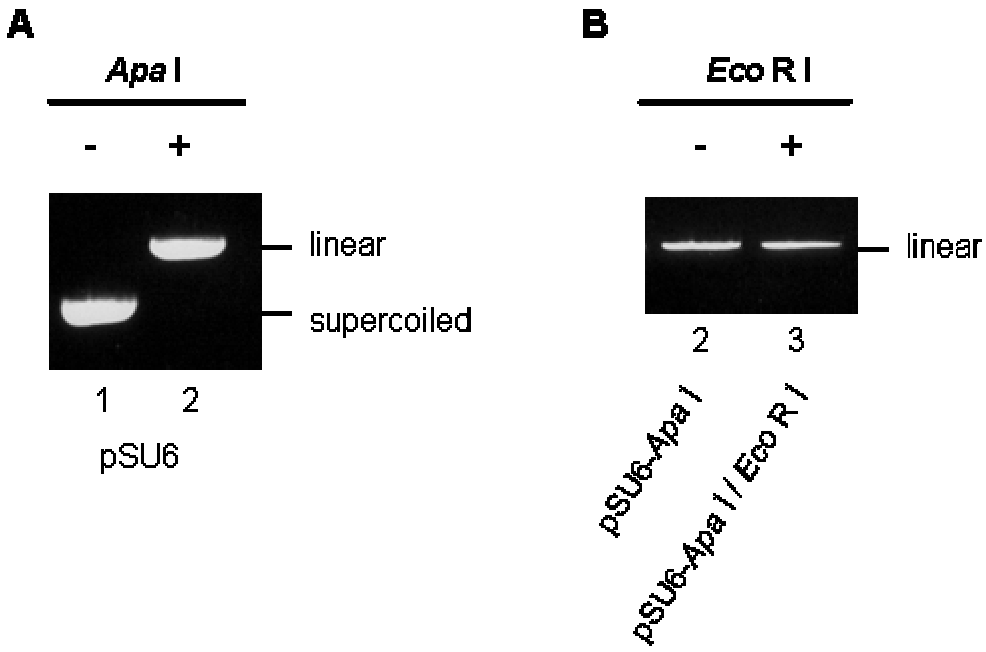


Figure 8: Digestion of pSU6 expression vector
pSU6 was linearized with *Apa* I (A) and subsequently digested with *Eco* R I (B). Analysis was by 1% agarose gel electrophoresis.

Three different potential siRNA sequences (A, B and C) targeting sites within the DNA binding domain of mouse PARP-1 were selected and the corresponding oligonucleotides were inserted into *Apa* I/*Eco* RI digested pSU6, as described under "Materials and Methods". One sequence complementary to a site within GAPDH mRNA was used as a positive functional silencing control. GAPDH protein is expressed in most mammalian cells at levels that can be readily detected by western blot analyses.

To select positive clones that contain siRNA inserts, plasmids purified from different clones were digested with *Hin*d III restriction enzyme: plasmids with the insert are not digested by *Hind* III, as the restriction site has been removed by the double *Apa* I/*Eco* R I digestion (see Fig. 5); on the contrary the original pSU6 is linearized by this treatment.

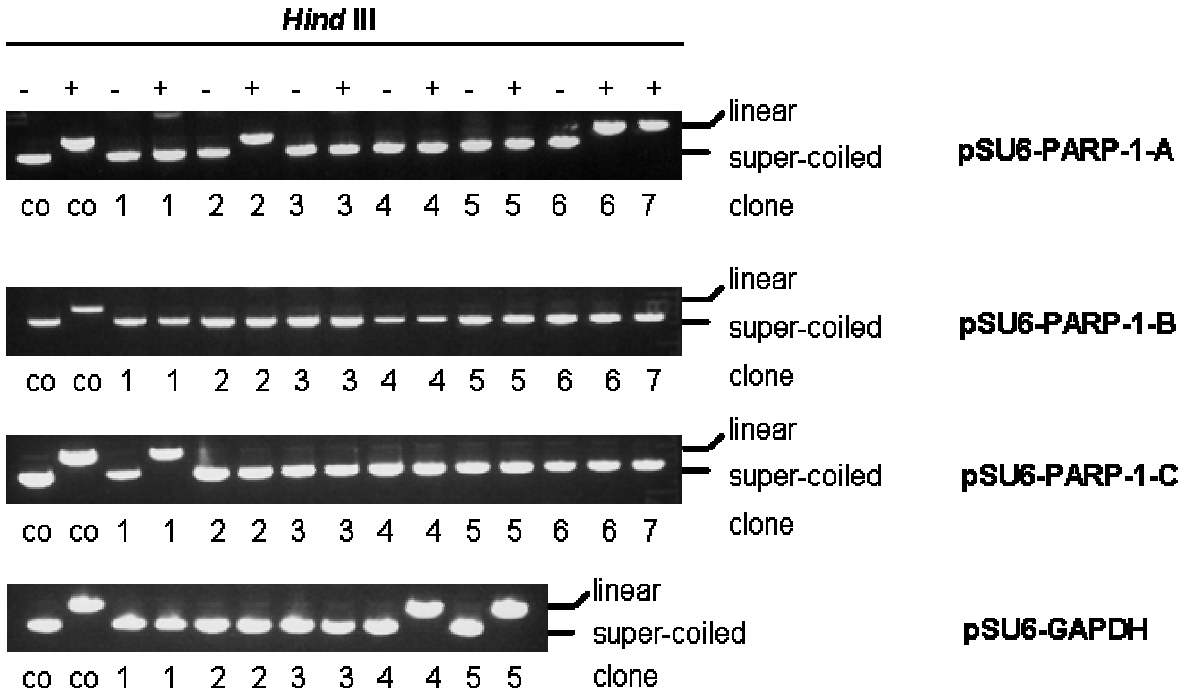


Figure 9: Selection of positive clones by restriction analysis.
Agarose gel electrophoresis of pSU6 (co), clones 1-7 of the three pSU6-PARP-1 constructs (A, B, C) and clones 1-5 of pSU6-GAPDH. For each pair of samples, the first lane corresponds to the undigested plasmid and the second one to the *Hind* III digested plasmid, lane 7 only digested plasmids are shown.

As shown in Fig. 9 clones No 1, 3, 4 and 5 of pSU6-PARP-1-A, all 7 clones of pSU6-PARP-1-B, clone No 2 – 7 of pSU6-PARP-1-C and clone No 1, 2 and 3 of pSU6-GAPDH were not digested by *Hind* III. Plasmids from these clones were amplified, purified and used in most of the following experiments.

4.1.2 Chemical transfection

In the last years many different chemical transfection reagents have been developed. In this study, I tested six different reagents for transfection of pSU6 silencing constructs in mouse embryonic fibroblasts. Three of them were based on Polyethylenimine (ExGen 500, JetPEI and the Transferrinfection method), one was based on liposome formulation (Lipofectamin), one on dendritic molecules (Polyfect Transfection Reagent) and one on repulsive membrane acidolysis technology (Metafectene Transfection Reagent). All reagents were used as recommended by the

manufacturers. To ensure high transfection efficiency, for all transfection reagents optimization was performed by varying the transfectant/DNA ratio.

Cells were transfected with either pSU6-GAPDH or pSU6-PARP-1 A, B or C. After 24 hours, whole cell lysates were prepared and analyzed by western blot; immunodetected bands were quantified by scanning densitometry. Equal loading was confirmed by immunodetection of either Tubulin (for pSU6-GAPDH transfected cells) or GAPDH (for pSU6-PARP-1 transfected cells).

As shown in Fig. 10, using six different chemical transfection reagents neither GAPDH nor PARP-1 expression could be downregulated to a significant extent: the maximum reduction of the targeted proteins was 10% of basal levels.

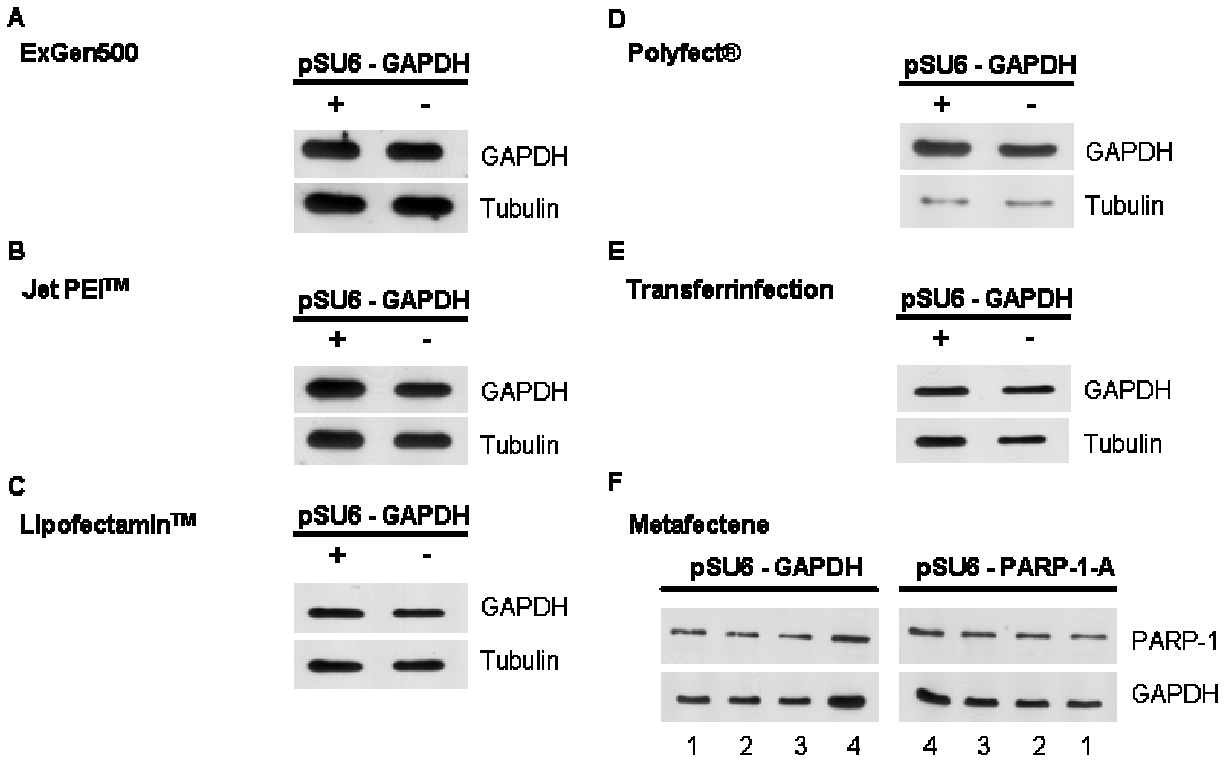


Figure 10: Effects of pSU6-GAPDH and pSU6-PARP-1-A chemical transfection in MEFs. Western blot analyses of whole cell lysates, antibodies against Tubulin, GAPDH and PARP-1 were used. (A)-(E): cells were either mock transfected (-) or transfected with pSU6-GAPDH (+); (F): cells were transfected with either pSU6-GAPDH (left panel) or pSU6-PARP-1-A (right panel) at different lipid:DNA ratios (lanes 1-3) and mock transfected cells (lane 4).

There are different possible reasons why protein silencing was unsuccessful in these experiments. First of all, efficient DNA transfection is a critical parameter. Thus,

one possibility could be that the uptake of vector DNA by the cells was very low. Another possibility is that the Polymerase III promoter is less active than expected. The third alternative is that the tested sequences are not good candidates for gene silencing by RNAi. However, this last possibility applies only for PARP-1 sequences, as the silencing potential of the GAPDH sequence was proven before by many research groups.

To determine whether the negative silencing results were due to low uptake of plasmid DNA by MEFs under the culture conditions used, a plasmid, 5.9 kbp in size, coding for GFP-p53 fusion protein, under the CMV immediate-early promoter, was chemically transfected. The GFP-p53 plasmid uptake was analyzed by indirect immunofluorescence of the expressed protein using anti-GFP antibodies. By transfecting the cells with ExGen 500 less than 5 % of cells took up the GFP-p53 plasmid, as indicated by the low number of stained nuclei (Fig. 11). All other tested reagents showed comparable low transfection efficiencies (data not shown).

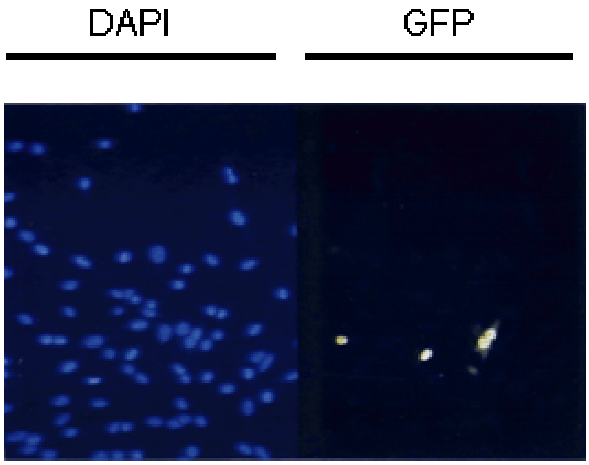


Figure 11: Indirect immunofluorescence of GFP-p53 in MEFs using anti-GFP antibodies (400 x magnification). Cells were transfected using the chemical transfection reagent ExGen 500. Left: DAPI staining of DNA; right: immunostaining with polyclonal antibody against GFP and FITC-conjugated anti-rabbit IgG. No background fluorescence was detectable in mock transfected cells.

4.1.3 Transfection of pSU6 by electroporation

Electroporation is a physical transfection method that involves the exposure of cells to a pulsed electric field which presumably creates pores in the plasma membrane. It has been used to introduce DNA into animal cells and has been successfully applied to a wide range of cell types which had not been accessible to other methods. Electroporation could therefore be the method of choice for efficient transfection of pSU6 in MEFs. To investigate the uptake of plasmid DNA by electroporation, the GFP-p53 plasmid was used and the expression of GFP-p53 was analyzed by indirect immunofluorescence using antibodies against GFP. As shown in Fig. 12 about 40 % of cells showed a nuclear staining, indicating uptake of plasmid and GFP-p53 expression.

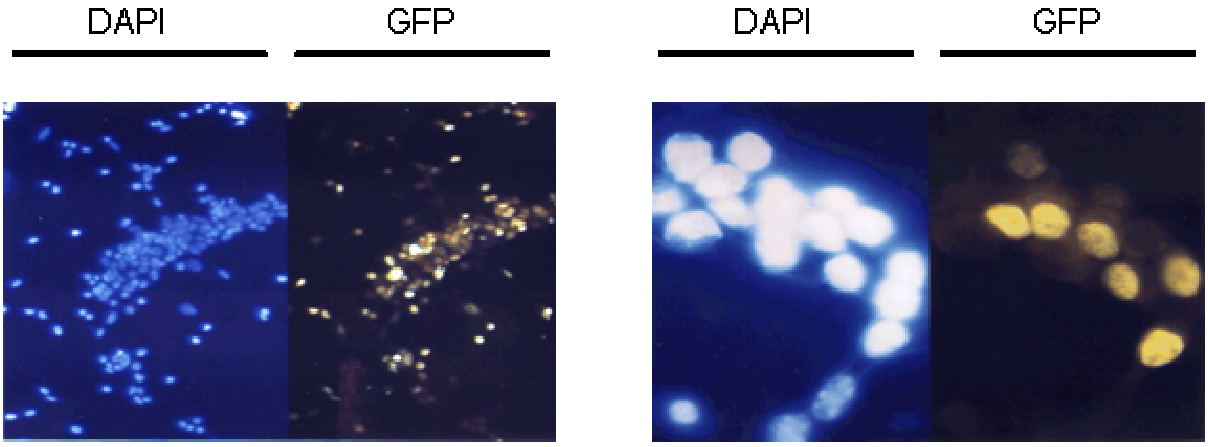


Figure 12: Indirect immunofluorescence of GFP-p53 transfected MEFs using anti GFP antibodies. Cells were transfected by electroporation. Left: 400 x magnification, right: 1000 x magnification (oil immersion). Blue: DAPI staining of DNA, yellow: indirect immunostaining of GFP-p53 by polyclonal anti-GFP antibody and FITC-conjugated anti-rabbit IgG.

In a next step, the suitability of electroporation to deliver siRNA-expression vectors was tested with the pSU6-GAPDH construct. As it is reported that addition of carrier DNA may increase transfection efficiencies, the vector was transfected either alone or in combination with salmon sperm (ss) DNA (0.23 µg/µl). In addition, a special plasmid purification kit was used to ensure endotoxin-free plasmid preparations. After 24 hours, whole cell lysates were analyzed by western blot and

scanning densitometry for quantification of GAPDH. GAPDH content was normalized to PARP-1 and the data were expressed as percent of the corresponding values for control cells (mock transfected cells). Results are shown in Fig. 13.

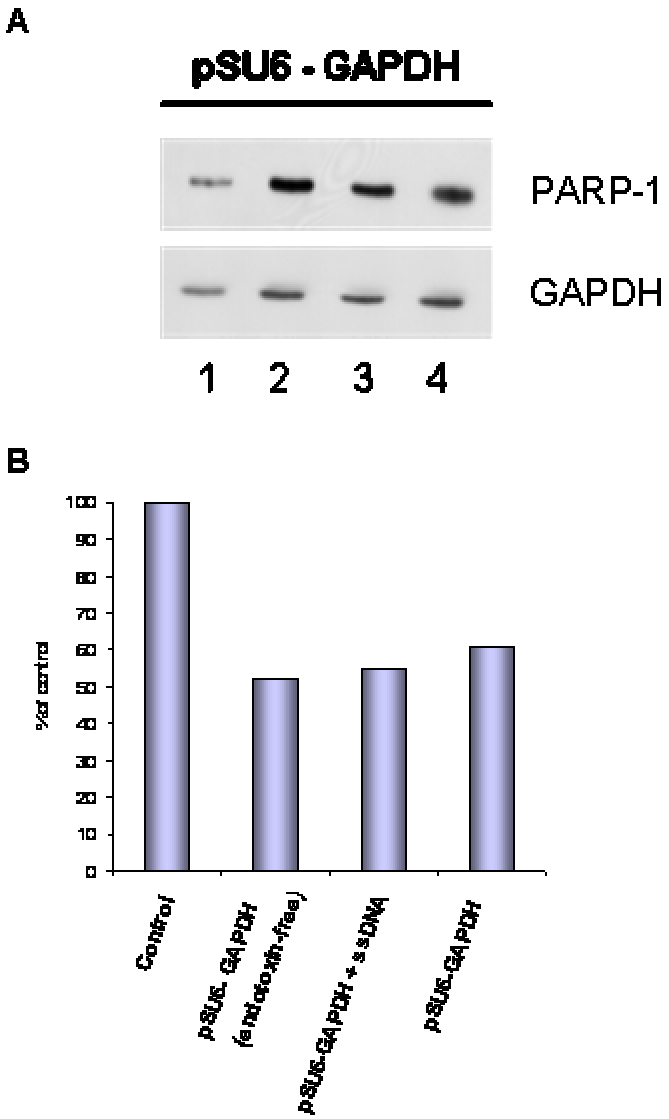


Figure 13: GAPDH levels in MEF`s transfected with pSU6-GAPDH by electroporation. (A): Western blot analysis of whole cell lysates. Lane 1: control cells, lane 2: pSU6-GAPDH (endotoxin-free), lane 3: pSU6-GAPDH + salmon sperm DNA, lane 4: pSU6-GAPDH. (B): quantification of the blot shown in A.

GAPDH protein levels were reduced by 40 % - 50 % in electroporated cells. The endotoxin-free plasmid gave slightly better results than the plasmid purified under standard conditions (45 % GAPDH reduction compared to 40 %). Also, a slight improvement was obtained by cotransfection of pSU6-GAPDH with carrier DNA.

4.2 Downregulation of PARP-1 expression using siRNAs

In this work, six different siRNAs with sequences matching distinct 21-nt coding regions within the DNA binding domain of PARP-1 (sequences A-F) were produced by in vitro transcription and purified as described under “Materials and Methods”. A GAPDH siRNA and scrambled PARP-1 sequence E were used as positive and negative controls, respectively. The quality of siRNAs at intermediate and final steps of the purification procedure was assessed by agarose gel electrophoresis. As an example, Fig. 14 shows the siRNA corresponding to PARP-1 sequence A, before and after a final digestion with single-strand specific ribonuclease to eliminate 5`-overhang sequences (see Methods, 2.9.3 for details).

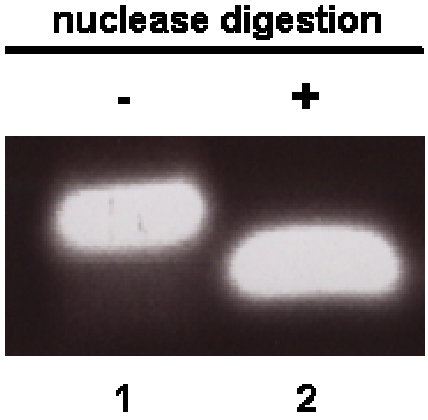


Figure 14: Agarose gel electrophoresis analysis of siRNA
siRNA before (lane 1) and after (lane 2) enzymatic digestion and purification.

The siRNA amount used for transfection is critical to the success of gene silencing experiments. Transfecting too much siRNA causes nonspecific reductions in gene expression and toxicity to the transfected cells. Transfecting too little siRNA does not change the expression of the target gene. The concentration of the siRNA preparations was determined on the basis of the absorbance at 260 nm and further confirmed by agarose gel electrophoretic analysis (Fig. 15). With the only exception of siRNA PARP-1 sequence A (lane 1), all other siRNAs had similar concentrations (lanes 2-7)

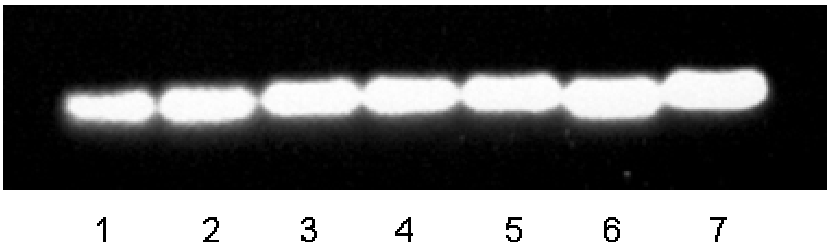


Figure 15: Electrophoretic analysis of *in vitro* transcribed and purified siRNAs
2.8 µg of PARP-1 siRNAs (A-F; lane 1-6) and GAPDH-siRNA (lane 7) were analyzed on 1.5 % agarose gel.

4.2.1 Silencing efficiency of different siRNAs

In vitro transcribed siRNAs were transfected into MEFs and the levels of PARP-1 and GAPDH protein were analyzed by Western blot and scanning densitometry 24 hours after transfection, numbers otherwise specified. An example of Western blot analysis is given in Fig. 16. Controls included cells that were either mock transfected or transfected with siRNA containing the same nucleotides as sequence PARP-1 E but in a randomly mixed order (PARP-1 E scrambled).

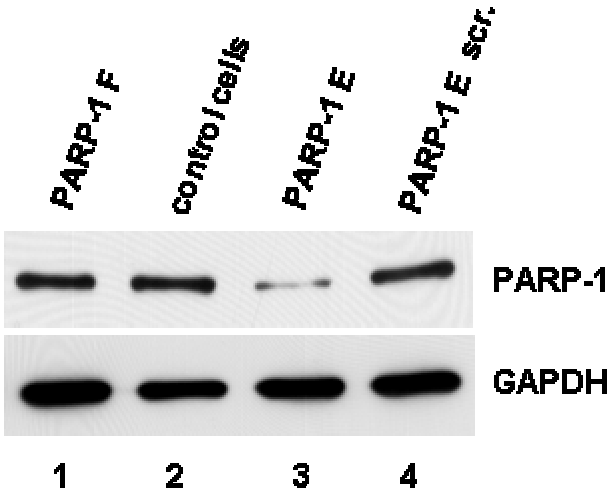


Figure 16: Western blot analysis of PARP-1 silencing in MEFs.
Whole cell lysates from mock transfected cells (lane 2) and cells transfected with PARP-1 sequence F (lane 1), PARP-1 sequence E (lane 3) and PARP-1 sequence E scrambled (scr.; lane 4).

Relative to mock transfection and PARP-1 E scrambled siRNA, all six PARP-1 siRNAs reduced PARP-1 protein content, albeit to a different extent (Fig. 17). PARP-1 C- and E-siRNAs appeared to be the most effective. In particular PARP-1 E siRNA reduced PARP-1 expression to nearly undetectable levels, whereas PARP-1 C siRNA reduced protein levels by about 65 %. PARP-1 siRNAs A, B, D and F induced a reduction of PARP-1 expression by 30 to 40 %. The positive control GAPDH siRNA reduced GAPDH expression by about 60 %. For any given sequence, variations in the level of silencing in different experiments may be due to different transfection efficiencies.

Taken together, this series of experiments demonstrate that PARP-1 E-siRNA is the best candidate for PARP-1 silencing.

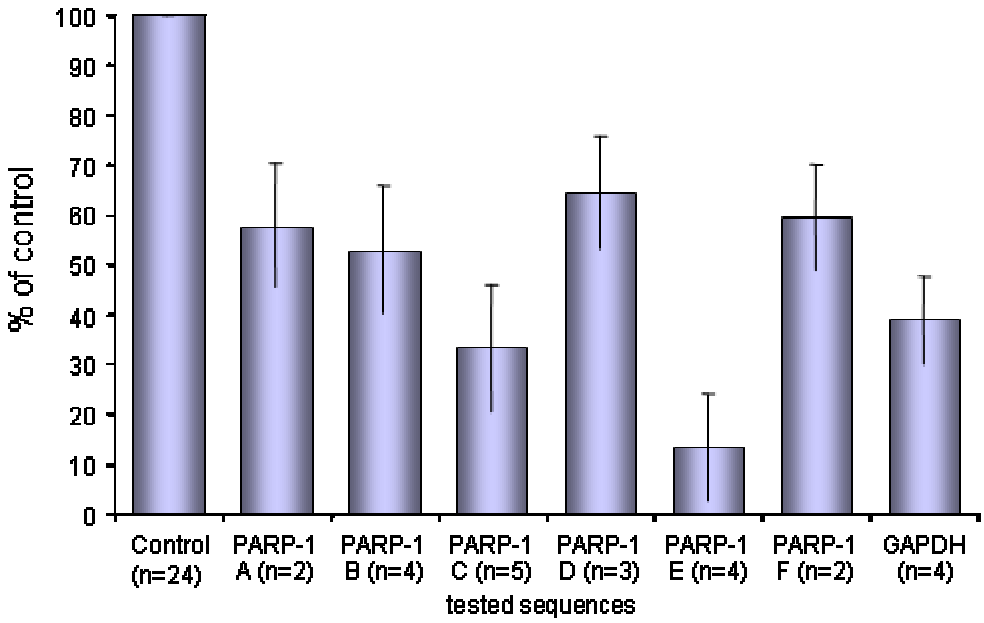


Figure 17: Silencing of PARP-1 and GAPDH by siRNA transfection
PARP-1 and GAPDH were detected by Western blot and quantified by scanning densitometry. PARP-1 content was normalized to GAPDH, while for GAPDH silenced cells, PARP-1 was used for normalization. Normalized data are expressed as % of the corresponding values for control (mock transfected) cells +/- SD.

4.2.2 siRNA dose-dependent silencing of PARP-1

When MEFs were transfected with PARP-1 E-siRNA at different concentrations (40 µM, 80 µM and 200 µM), similar reduction of PARP-1 levels was observed (Fig. 18, compare lanes 1, 2 and 3). Therefore 40-80 µM siRNA concentrations were chosen for further experiments. Compared to mock transfected control cells, the inhibition of PARP-1 expression by PARP-1 E-siRNA was calculated to be higher than 60% (Fig. 18, compare lane 1, 2 and 3 with lane 4). The lack of complete inhibition may be the consequence of low transfection efficiency in this experiment.

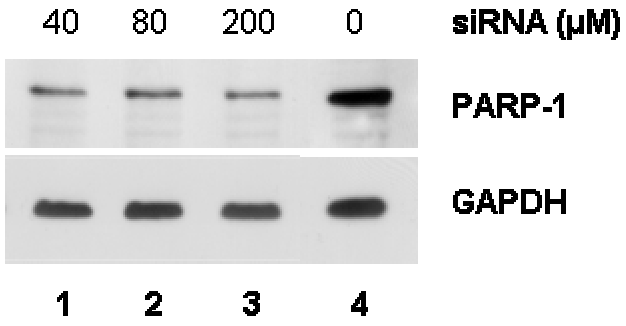
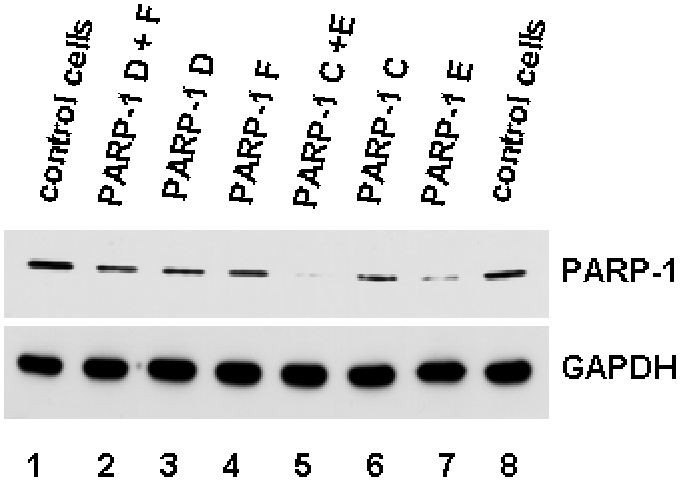


Figure 18: PARP-1 silencing by different amounts of siRNA
Whole cell lysates from MEFs transfected with increasing amounts of PARP-1 E siRNA were analyzed by Western blot using anti-PARP-1 or anti-GAPDH antibodies.

4.2.3 Effect on PARP-1 expression of combined siRNAs

The use of a single species of siRNA to target mRNA is not a biologically relevant situation, as cells would normally encounter multiple siRNAs produced from long dsRNA by the RNase III-like DICER. I asked whether the complexes involved in cleaving the mRNA might have evolved to utilize multiple siRNA species, for instance through a mechanism of allosteric stimulation of RISC, and thus work more efficiently with multiple siRNAs. Specifically, I wanted to know whether combinations of siRNAs would result in increased activity through synergism or addition. Testing this concept by co-transfecting various combinations of two siRNAs in MEFs, no additive or synergistic effect could be discerned by western blot analyses (Fig. 19). Co-transfection of two semipotent sequences (PARP-1 D and F) together did not cause any further silencing then each of them separately (compare lane 2 with lanes 3 and 4). Co-transfecting one active (E-siRNA) and one less-active C-siRNA did not reduce the silencing potential of the very potent E-siRNA (compare lane 5 with lanes 6 and 7).

A



B

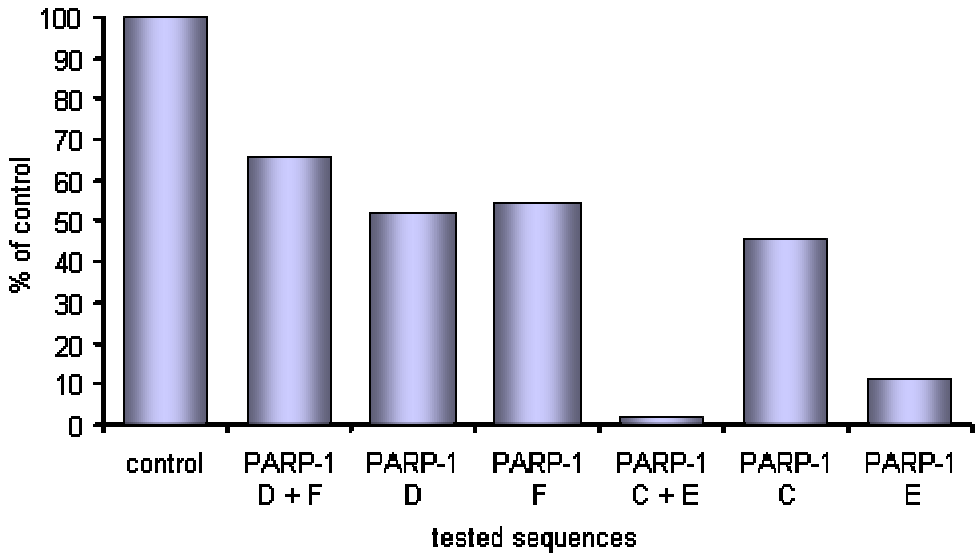


Figure 19: Downregulation of PARP-1 expression using combined siRNAs. (A): Western blot analysis of PARP-1 and GAPDH in whole cell lysates from control (mock transfected) cells (lane 1 and 8) and cells either transfected with one PARP-1 siRNA alone (lane 3, 4, 6 and 7), or transfected with combinations of PARP-1 siRNAs (lane 2 and 5). (B): Quantification of the Western blot shown in A.

4.2.4 Time course of PARP-1 silencing by siRNAs

A crucial aspect of RNAi experiments is the time required to reduce protein expression below the threshold level that is critical to sustain normal protein function. As shown in Fig. 20, PARP-1 content in MEFs was reduced to undetectable levels already 24 hours after PARP-1 E-siRNA transfection.

On the other hand, the approach of gene silencing by direct transfection of siRNAs into mammalian cells is limited by the transient nature of the silencing effect as the siRNAs are turned over by the cell. Western blot analysis of MEFs harvested 24, 48 and 72 hours after transfection with PARP-1 E-siRNA showed that the silencing effect lasts at least up to 72 hours (Fig. 20).

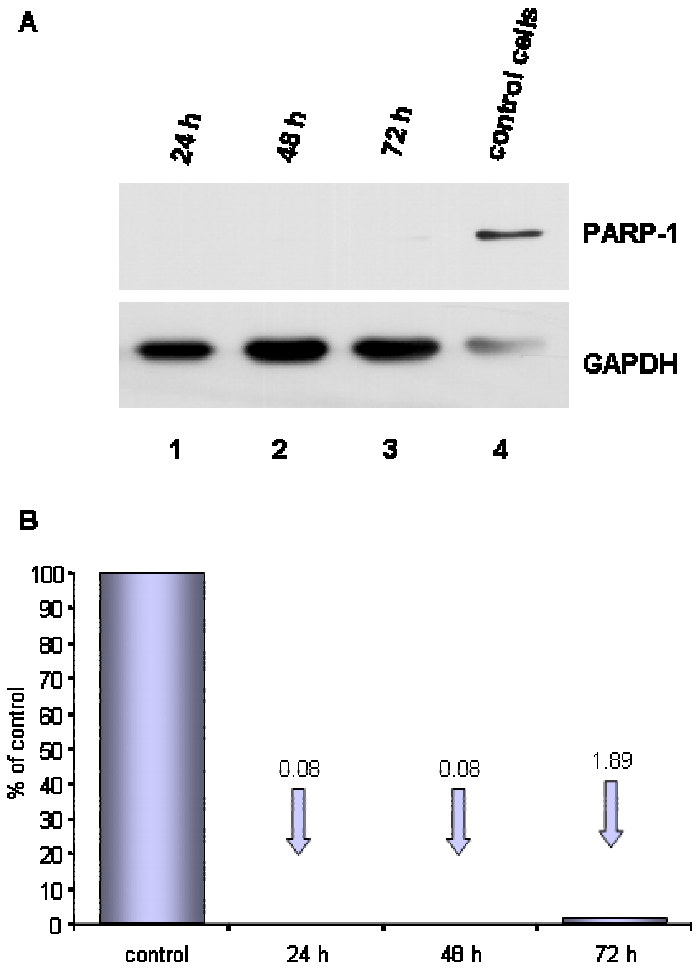


Figure 20: Time course of PARP-1 silencing (A): Western blot analysis of whole cell lysates from control (mock transfected) cells (lane 4) and PARP-1 E-siRNA transfected cells harvested 24, 48 and 72 hours after transfection (lane 1-3). (B): Quantification of the Western blot shown in A.

4.2.5 Response to oxidative stress of PARP-1 wild-type and PARP-1-deficient cells

MEFs were treated with 200 µM H_2O_2 as described in "Materials and Methods" and observed under a phase contrast microscope. This treatment had different effects, depending on the PARP-1 status of the cells.

As shown in Fig. 21, PARP-1 wild type cells appeared to be very sensitive to oxidative stress and after 30 minutes of incubation at 37 °C in the presence of H_2O_2 most of them detached from the plate while the remaining cells exhibited a rounded shape. In contrast, PARP-1 silenced cells (E-siRNA, 24 hours after transfection) as well as PARP-1 knock-out cells resisted this treatment; thus confirming PARP-1 involvement in oxidative stress induced cytotoxicity.

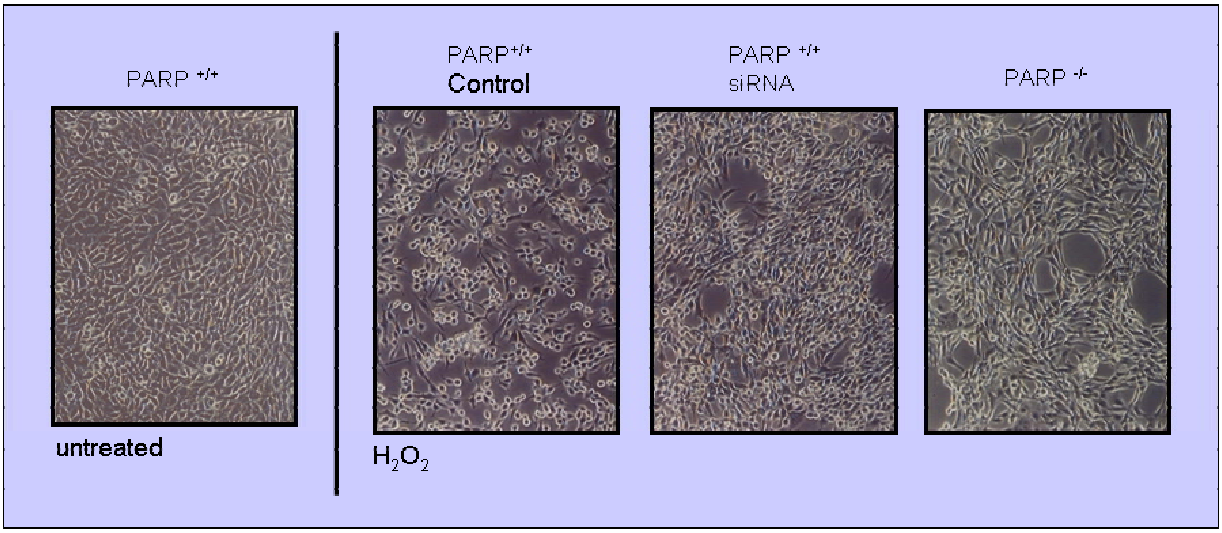


Figure 21: Effect of H_2O_2 in the presence and absence of PARP-1. Phase contrast microscopy (20 x objective).

5. DISCUSSION

5.1 Comparison of strategies to induce RNAi

Over the last couple of years a large variety of techniques have emerged that make dsRNA-induced silencing applicable to mammalian cells. Each is associated with specific advantages and disadvantages (Table 1).

TABLE 1

Advantages and Disadvantages of Different siRNA Delivery Strategies

	Advantages	Disadvantages
Chemical and *in vitro* enzymatic synthesis	Rapid synthesis RNAse III: produces siRNA mixtures, obviating need for testing the efficacy of siRNAs separately High purity using chemical synthesis	Transient RNAi Purity and specificity using enzymatic synthesis is variable Chemical synthesis expensive for multiple siRNAs Transfection-dependent
DNA plasmid vector or Cassette	More economical for multiple sequences Stable RNAi achievable using selection marker	More labor intensive to generate Transfection-dependent
Virus-mediated	May be effective in cells resistant to transfection with dsRNA and plasmids Integration produces stable RNAi even in the absence of a selection pressure	More labor intensive to generate Potential biohazard

(Duxbury et al. 2004)

In the work presented here, I set up conditions for PARP-1 gene silencing in MEFs using two different strategies: one approach involved the use of polymerase III promoter-based DNA plasmids which express siRNA within the cells, while the second was based on the direct transfection of *in vitro* transcribed siRNAs.

The siRNA expression vector system

Using this approach, siRNAs are produced by polymerase III promoter-based DNA plasmids or expression cassettes (Sui et al. 2002). These constructs produce small inverted repeats, separated by a spacer of three to nine nucleotides, termed short hairpin RNAs (shRNAs), which are processed by Dicer into siRNAs (Paddison et al. 2002). Transcription begins at a specific initiation sequence, determined by the promoter used. In addition to a defined initiation sequence, the U6 polymerase III promoter terminates at TTTT or TTTTT (Bogenhagen et al. 1980), giving as a product shRNAs that contain a series of uridines at the 3` end, a characteristic which appears to facilitate RNAi (Elbashir et al. 2001b).

Although, the vector based system is more labor intensive to generate, it was chosen first because of the higher stability of DNA plasmid relative to RNA molecules and because RNAi can be sustained for as long as 2 months post-transfection (Brummelkamp et al. 2002a).

While sufficient gene silencing using U6-expression vector systems has been achieved by many groups, in the experiments reported in this thesis, using different chemical transfection methods in mouse embryonic fibroblasts, the system has failed to give appreciable downregulation of PARP-1 as well as of GAPDH (Fig. 10).
In general three reasons are conceivable regarding inefficient silencing: 1. Tested sequences ineffective to induce RNAi, 2. Low activity of the Polymerase III promoter; 3. Low transfection efficiencies of expression vectors.

The three different PARP-1 sequences (A, B and C) have been chosen randomly among potential target sequences in the region coding for the DNA binding domain of PARP-1. It was the aim of this work to set up conditions for effective RNAi of PARP-1, which includes the identification of suitable target sequences. Thus, it cannot be excluded that the tested sequences failed to reduce PARP-1 protein levels because they are simply not good candidates for RNAi. However, this was certainly not the case for the GAPDH-siRNA. In fact, the siRNA used with a sequence derived

from the 5`medial region of the GAPDH mRNA, has been shown to reduce the expression of GAPDH by 50-95 % in several mammalian cell lines (Brown et al. 2002), and is generally used as a positive control in siRNA experiments. Using different chemical transfection reagents and the pSU6-GAPDH, I did not find any appreciable downregulation of GAPDH. Hence it must be assumed that either transfection was inefficient or that in mouse embryonic fibroblasts the polymerase III promoter is less active than expected. As the polymerase III promoter has been shown to successfully knock down the expression of cdk-2 and lamin A/C in diverse cell lines (Sui et al. 2002), low transfection efficiencies are the most probable reason for the observed absence of downregulation.

This conclusion is supported by the results obtained in an independent series of experiments in which transfection efficiencies were evaluated by using a GFP-p53 expression vector and indirect immunofluorescence with an anti-GFP antibody. Results showed that a maximum of 5 % of the cells took up the plasmid regardless of the transfection reagent used. Better transfection efficiencies could be obtained by electroporation, a method that involves the exposure of cells to a pulsed electric field under controlled conditions: in this case up to 40 % of the cells took up the plasmid and expressed GFP-p53 (Fig. 12). Accordingly, electroporation of pSU6-GAPDH resulted in about 50 % reduction of GAPDH (Fig. 13). The increase in the silencing effect using electroporation in comparison to chemical transfection is in line with reports that show that physical transfection methods can be successfully used to introduce DNA in cell types which have not been accessible to other transfection methods (Chu et al. 1987).

Recently, expression vectors with antibiotic selectable markers have become available: in these cases transient antibiotic selection permits enrichment of the cells that have received the plasmid, thus compensating for low transfection efficiencies. These vectors may be used in the future in alternative to pSU6 for PARP silencing.

siRNAs

In contrast to expression vectors, *in vitro* transcribed siRNA molecules can be easily transfected into mammalian cells. The strategy of *in vitro* enzymatic synthesis of siRNAs is becoming increasingly popular because this method is rapid and may be the best approach for initial "proof of principle" experiments as well as siRNAs synthesized by industrial chemical processes (Micura 2002).

The method relies on the transcription of specific oligonucleotides by the T7 phage polymerase (Donze and Picard 2002). The polymerase produces individual RNA molecules, sense and antisense strands, which, when annealed, form siRNAs. Extra nucleotides required by the T7 promoter are removed by RNase digestion and cleaning steps.

In this thesis, six different siRNA with sequences matching the DNA binding domain of PARP-1 were synthesized and tested for their ability to downregulate PARP-1 expression. PARP-1 E-siRNA has proven to be the best candidate for gene silencing, as it reduced PARP-1 content to undetectable levels (Fig. 20).

siRNAs targeting different positions on PARP-1 mRNA differed in their silencing potency, with reduction of PARP-1 ranging from 35 to 98 % of basal levels (Fig. 17). This feature can be exploited experimentally to modulate, rather than suppress, PARP-1 expression into the cells.

An important point in experiments is the time required to reduce protein expression below the threshold level that is critical to sustain normal protein function. This is in large part determined by the efficiency of the siRNA to target the mRNA of choice. But in addition, protein stability is a critical factor. The time required to reduce protein expression below the critical level, once the mRNA is degraded or translation is shut off, is primarily determined by the half-life of that protein. PARP-1 is a protein with a relatively short half-life. In fact, with the most potent siRNA, the maximal effect was observed already after 24 hours.

An important factor that has to be considered is the transient nature of the silencing effect obtainable by siRNA transfection. As the siRNAs are turned over by the cell, the silenced genes can recover in time, limiting this approach to analysis for short time periods (Duxbury et al. 2004).

Western blot analysis of MEFs harvested 24, 48 and 72 hours after the start of transfection with PARP-1 E siRNA showed that indeed the silencing effect lasts at least up to 72 hours (Fig. 20).

5.2 PARP-1 knockdown and oxidative stress

The PARP-1 suicide hypothesis suggests that massive DNA damage, which occurs with severe oxidative stress, may trigger the activation of PARP-1 to such an extent that all the cellular NAD^+ may be depleted (Berger et al. 1983). Thus, the cellular energy supply may become deficient and this may lead to functional and morphological alterations which may result in cell death. Studies with PARP-1 knockout mice as well as PARP inhibitors confirm the role of PARP-1 in oxidant-mediated cell death (Chiarugi 2002).

In fact, the absence of PARP-1 activity protects cells against the cytotoxic effects of oxidizing agents.

To functionally validate PARP-1 silencing by RNAi, PARP-1 silenced cells were treated with H_2O_2 for 30 minutes and compared with wild-type and PARP-1 knockout cells receiving the same treatment. PARP-1 silenced cells, as well as PARP-1 knockout cells, proved to be more resistant to oxidative stress (Fig. 21).

In conclusion, having established conditions for effective PARP-1 silencing using the siRNA transfection method, these studies will provide an essential background for further investigations. In particular, the acquired know-how will facilitate the identification and production of siRNA targeting other components of the PARP family. An interesting candidate is obviously PARP-2, which shares with PARP-1 a “guardian of the genome” function, but is most probably involved also in other vital processes, as suggested by studies with knockout mice.

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7. SUMMARY

Poly(ADP-ribose) polymerases (PARPs) constitute a family of related proteins that play diverse roles in various cellular functions, and share a highly homologous catalytic domain with a common enzymatic activity: the synthesis of variously sized ADP-ribose polymers, covalently bound to acceptor proteins. In this reaction, the respiratory coenzyme nicotinamide adenine dinucleotide (NAD^+) serves as a source of ADP-ribose moieties.

This thesis work was aimed at setting up conditions for specific silencing of individual PARPs in living cells using RNA interference (RNAi). RNAi is a recently developed technique that has proven to be a powerful tool for rapid and specific downregulation of target genes. PARP-1, the most abundant and best characterized member of the PARP family, was chosen as an initial target.

To knock-down PARP-1 in mouse embryonic fibroblasts (MEFs), two approaches out of the various techniques for RNAi were tested. One approach involved the use of polymerase III promoter-based DNA plasmids which express siRNA within the cells. Even though it is labor intensive, this system has the advantage of allowing RNAi over a long time period. A disadvantage is that the success of this strategy suffers from the low transfection efficiency of DNA plasmids. The second approach was based on the direct transfection of *in vitro* transcribed siRNAs; siRNA molecules can be easily transfected into mammalian cells but, on the other hand, RNAi can be obtained only transiently.

In regard to the first approach, three sequences targeting different regions in the PARP-1 gene were inserted in the pSilencer 1.0-U6 vector (pSU6) and transfected into MEFs using different chemical transfection methods. A plasmid expressing siRNA targeting the glycolytic enzyme GAPDH was used as a positive control. As monitored by immunoblotting, the system failed to give appreciable downregulation of PARP-1, nor of GAPDH. On the basis of an independent series of experiments, in which a GFP-p53 expression vector was chemically transfected into MEFs, the failure of gene silencing could be attributed to low transfection efficiencies. Better transfection efficiencies could be obtained using electroporation, by which 40 % of the cells took up the plasmid and expressed GFP-p53. Accordingly, 40-50 % reduction of GAPDH cellular content could be achieved by electroporation of the specific siRNA-pSU6 construct.

In regard to the second approach, six different siRNAs with sequences matching the DNA binding domain of PARP-1 were synthesized *in vitro* and tested for their ability to downregulate PARP-1 expression. Immunoblotting analyses revealed that siRNA molecules, targeting different positions on PARP-1 mRNA, were able to reduce PARP-1 expression from 35 % to more than 98 % of basal levels within 24 hours and that the silencing effect persists at least up to 72 hours after transfection.
As expected, and in agreement with data from mice knockouts, post-transcriptional silencing of PARP-1 by RNAi did not cause any obvious phenotype under normal growth conditions; however, silenced cells appeared to be more resistant against oxidative stress than wild-type cells.

In conclusion, these studies establish conditions for effective PARP-1 silencing in cultured cells; this will provide an essential background for further investigations on the role of PARP-1 and, more generally, of poly (ADP-ribose) synthesis, in the response to DNA damage. The acquired know-how will facilitate the identification and production of siRNA targeting other components of the PARP family.

8. ZUSAMMENFASSUNG

Die Familie der Poly(ADP)polymerasen (PARPs) bezeichnet verwandte Proteine, die unterschiedliche Rollen bei verschiedenen zellulären Funktionen spielen. Allen PARP Proteinen gemeinsam ist eine stark homologe Domäne mit enzymatischer Aktivität, die die Synthese von ADP-Ribose Polymeren verschiedener Größe katalysiert. Die Polymere werden kovalent an Akzeptorproteine gebunden, wobei in der Reaktion das respiratorische Koenzym Nikotinamid-Adenin-Dinukleotid (NAD^+) als Substrat für die ADP-Ribose-Einheiten dient.

Ziel der vorliegenden Arbeit war es, ein System zu entwickeln, dass die Downregulation von PARP-Proteinen mittels RNA Interferenz (RNAi) in Zellkulturen ermöglicht. RNAi ist eine vor kurzem entwickelte Technik, die zu einer schnellen und spezifischen Downregulation von Genen führt. Die notwendigen Bedingungen für ein effizientes Gen Silencing wurden zunächst für PARP-1, das am häufigsten vorkommende und am besten charakterisierte Enzym der PARP-Familie, ermittelt.

Die Verminderung der PARP-1 Proteinmenge in Fibroblasten von der Maus wurde mittels zweier unterschiedlicher Techniken von RNAi getestet. Die erste Methode basiert auf der Verwendung von DNA Plasmiden. Unter der Kontrolle von Polymerase III Promotoren werden die siRNA Moleküle in den Zellen exprimiert. Auf der einen Seite ist diese Methode sehr arbeitsintensiv, andererseits hat sie den Vorteil, dass der RNAi Effekt über eine längere Zeitperiode erreicht werden kann. Der Hauptnachteil dieser Methode ist jedoch, dass der Erfolg von der Transfektionseffizienz der DNA Plasmide abhängig ist. Die zweite Methode, die in dieser Studie verwendet wurde, basiert auf der direkten Transfektion von *in vitro* hergestellter siRNA; siRNA Moleküle können leicht in Säugetierzellen transfiziert werden, haben aber auf der anderen Seite den Nachteil, dass nur ein zeitlich begrenzter RNAi Effekt erzielt werden kann.

Hinsichtlich der ersten Methode wurden drei Sequenzen, die unterschiedliche Regionen im PARP-1 Gen kodieren, in den pSilencer 1.0-U6 Vektor (pSU6) eingebaut und mit Hilfe von verschiedenen chemischen Methoden in Mausfibroblasten transfiziert. Zudem wurde ein Plasmid, das siRNA Moleküle exprimiert, die spezifisch für das glykolytische Enzym GAPDH sind, als positive Kontrolle verwendet. Wie mittels Immunoblotting gezeigt werden konnte, wurde mit diesem System weder eine ausreichende Verminderung der PARP-1 Protein Menge,

noch derjenigen von GAPDH, erreicht. Auf der Basis einer unabhängigen Serie von Versuchen, bei denen ein GFP-p53 Expressionsvektor chemisch in Fibroblasten von der Maus transfiziert wurden, konnte das Ausbleiben des Gen-Silencing auf niedrige Transfektionsraten zurückgeführt werden. Höhere Raten dagegen zeigte die Transfektion mittels Elektroporation. Bei Verwendung dieser Technik nahmen 40 % der Zellen das Plasmid auf und exprimierten GFP-p53. Dementsprechend konnte eine 40-50 %ige Verminderung der zellulären GAPDH Menge durch Elektroporation des spezifischen siRNA-pSU6 Konstrukts erreicht werden.

Im Hinblick auf die zweite Methode wurden sechs siRNAs Sequenzen aus der DNA-Bindungsdomäne von PARP-1 *in vitro* synthetisiert und auf ihr Vermögen die PARP-1 Expression zu vermindern hin untersucht. Immunoblot Analysen zeigten, dass die siRNA Moleküle, die für verschiedene Positionen auf der mRNA von PARP-1 kodieren, in der Lage waren, die PARP-1 Expression von 35 % bis mehr als 98 % der ursprünglichen Level zu reduzieren. Der Effekt wurde nach 24 Stunden sichtbar und hielt bis mindestens 72 Stunden nach der Transfection an.

Wie erwartet und in Übereinstimmung mit Daten von Knockout Mäusen zeigte das posttranskriptionale Silencing von PARP-1 mittels RNAi unter normalen Wachstumsbedingungen keinen offensichtlichen Phenotyp; jedoch waren die mit siRNA behandelten Zellen resistenter gegenüber oxidativem Stress als die Wildtyp Zellen.

Die vorliegende Studie zeigt die notwendigen Bedingungen für ein effizientes Silencing von PARP-1 in Zellkulturen; diese Methode wird die Basis für weitere Untersuchungen sein, die letztendlich zur Identifikation der Funktion von PARP-1, und mehr allgemein, zur Funktion der Poly(ADP-Ribose) Synthese, als Antwort auf DNA Schäden, führen. Das erworbene Wissen wird das Identifizieren und die Herstellung von siRNA Molekülen, die die weiteren Mitglieder der PARP Familie kodieren, stark erleichtern.

9. CURRICULUM VITAE

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